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(57) Abstract

An expression vector for expressing a protein or polypeptide in a bacterium, which comprises a first DNA sequence encoding at least a secretion signal of a lipoprotein, and a second DNA sequence encoding a protein or fragment thereof, or polypeptide or peptide heterologous to the bacterium which expresses the protein or fragment thereof, or polypeptide or peptide. The bacterium expresses a fusion protein of a lipoprotein or lipoprotein segment and the protein or fragment thereof, or polypeptide or peptide heterologous to the bacterium which expresses the protein or fragment thereof, or polypeptide or peptide. Such expression vectors increase the immunogenicity of the protein or fragment thereof, or polypeptide or peptide by enabling the protein or fragment thereof, or polypeptide or peptide to be expressed on the surface of the bacterium. Bacteria which may be transformed with the expression vector include mycobacteria such as BCG. The expression vectors of the present invention may be employed in the formation of live bacterial vaccines against Lyme disease wherein the bacteria express a surface protein of *Borrelia burgdorferi*, the causative agent of Lyme disease.

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BACTERIAL EXPRESSION VECTORS CONTAINING DNA ENCODING SECRETION SIGNALS OF LIPOPROTEINS

This application is a continuation-in-part of application Serial No. 780, 261, filed October 21, 1991.

This invention relates to expression vectors for expressing a protein in a bacterium, such as for example, a mycobacterium. More particularly, this invention relates to expression vectors for expressing and secreting proteins which are heterologous to the bacterium which expresses such proteins wherein such vectors further include DNA encoding at least the secretion signals of lipoproteins designed to achieve lipid acylation and surface expression of heterologous proteins.

Certain mycobacteria represent major pathogens of man and animals. For example, tuberculosis is generally caused in humans by Mycobacterium tuberculosis, and in cattle by Mycobacterium bovis, which may also be transmitted to humans and other animals. Mycobacteria leprae is the causative agent of leprosy. M.tuberculosis and mycobacteria of the avium-intracellulare-scrofulaceum group (MAIS group) represent major opportunistic pathogens of patients with acquired immune deficiency syndrome (AIDS). M.pseudotuberculosis is a major pathogen of cattle.

On the other hand, Bacille Calmette-Guerin, or BCG, an avirulent strain of M.bovis, is widely used in human vaccines, and in particular is used as a live vaccine, which is protective against tuberculosis. BCG is the only childhood vaccine which is currently given at birth, has a very low incidence of adverse effects, and can be used repeatedly in an individual. (eg., in multiple forms). In addition, BCG and other mycobacteria (eg., M.smegmatis), employed in vaccines, have adjuvant properties among the best currently known and, therefore, stimulate a recipient's immune system to respond to antigens with great effectiveness.

It has been suggested by Jacobs, et. al, Nature, Vol. 327, No. 6122, pgs. 532-535 (June 11, 1987), that BCG could be used as a host for the construction of recombinant vaccines. In other words, it was suggested to take an existing vaccine (in this case against tuberculosis) and expand its protective repertoire through the introduction of one or more genes from other pathogens.

Transformation, the process whereby naked DNA is introduced into bacterial cells, has been carried out successfully in mycobacteria. Jacobs, et al (1987), as hereinabove cited, have described transformation of mycobacteria by electroporation. Electroportation can give from 10^5 to 10^6 transformants per μg of plasmid DNA and such plasmid DNA's may carry genes for resistance to antibiotic markers such as kanamycin, Snapper, et al, PNAS, Vol. 85, pgs. 6987-6991 (September, 1988); to allow for selection of transformed cells from non-transformed cells.

Jacobs, et al (1987) and Snapper, et al (1988) have also described the use of cloning vehicles such as plasmids and bacteriophages, for carrying genes of interest into mycobacteria.

Lee, et al., PNAS, Vol. 88, pgs. 3111-3115 (April 1991), describe vectors which employ DNA encoding a mycobacterial phage integrase and phage attachment site to effect site-specific integration into a mycobacterial chromosome. Such vectors permit stable integration of vectorss encoding foreign antigen genes into a mycobacterial chromosome.

Stover, et al., (Nature, Vol. 351, pgs. 456-460 (June 6, 1991)) describe integrative and extrachromosomal vectors employing mycobacterial HSP60 and HSP70 promoters to express foreign antigens cytoplasmically in recombinant BCG. Stover, et al. demonstrated that recombinant BCG expressing foreign antigens with these vectors could be used as immunogens to generate humoral and cellular immune responses to the foreign antigens.

Combination of the above-mentioned techniques, along with standard tools of molecular cloning (e.g., use of restriction enzymes, etc.) allows the cloning of genes of interest into vectors and introduction of such genes into mycobacteria.

In accordance with an aspect of the present invention, there is provided an expression vector for expressing a protein or polypeptide or peptide in a bacterium. The expression vector comprises a first DNA sequence encoding at least a secretion signal of a lipoprotein; and preferably further comprises a second DNA sequence encoding a protein or fragment thereof or polypeptide or peptide heterologous to the bacterium which expresses the protein or fragment thereof, or polypeptide or peptide, whereby the bacterium expresses a fusion protein of a lipoprotein or lipoprotein segment (which may include the secretion signal), and the protein or fragment thereof, or polypeptide or peptide heterologous to the bacterium which expresses the protein or polypeptide or peptide.

Such an expression vector may be employed in any of a variety of bacteria which may be employed in vaccines, including live vaccines. In particular, in one embodiment, the bacterium is a mycobactrium such as, but not limited to, Mycobacterium bovis - BCG, M.smegmatis, M.avium, M.phlei, M.fortuitium, M.lufu, M. paratuberculosis, M.habana, M.scrofalaceum, M.intracellulare, and M. vaccae.

In one embodiment, the mycobacterium is M.bovis-BCG.

Although the scope of the present invention is not to be limited to any theoretical reasoning, it is believed that the signal sequence of the lipoprotein enables the expressed recombinant fusion protein to be modified such that the protein is expressed at the surface of the bacterium as a chimeric lipoprotein. For example, the fusion protein may include processing or recognition site(s) for signal peptidase II in the signal sequence portion, which enables lipid acylation of the

fusion protein. Such lipid acylation of the fusion protein may enhance the immunogenicity of the heterologous protein or fragment thereof, or polypeptide or peptide portion of the fusion protein. Also, the signal sequence enables the fusion protein to be expressed at and anchored to the surface of the bacterium, thus making the heterologous protein or polypeptide more accessible, which also may increase the immunogenicity of the protein or fragment thereof, or polypeptide or peptide. Also, because such fusion proteins may be expressed on the surface of the bacterium, such expression or secretion of the fusion protein will permit the expression of antigens which may be lethal if expressed or maintained cytoplasmically in the bacterium. It is to be understood that the heterologous protein or fragment thereof, or polypeptide or peptide may itself be a lipoprotein, such as the OspA antigen of Borrelia burgdorferi, which is hereinafter discussed, or a non-lipoprotein, such as, for example, HIV antigens, tetanus toxoids, diphtheria toxoids, cholera toxoids, pertussis toxoids, and malarial antigens. Thus, the expression vectors of the present invention enable the genetic engineering of a non-lipoprotein moiety which may become anchored to the surface of a bacterium.

Thus, the expression vectors enable the expression of heterologous genes or gene segments (which originally encoded non-lipoproteins) as chimeric surface lipoproteins. This is accomplished by gene fusion of the foreign genes or gene segments to vector encoded genes or gene segments encoding lipoproteins or lipoprotein signal peptides, respectively.

In one embodiment, the first DNA sequence encodes at least a secretion signal of a mycobacterial lipoprotein. The mycobacterial lipoprotein may, in one embodiment, be an M.tuberculosis lipoprotein. The M. tuberculosis lipoprotein may be selected from the group consisting of the M.tuberculosis 19 kda antigen and the M.tuberculosis 38 kda antigen.

Other lipoproteins, of which at least the secretion signal may be encoded by the first DNA sequence include, but are not limited to, Braun's lipoprotein of E. coli, S. marcescens, E. amylosora, M. morganii, and P. mirabilis, the TraT protein of E. coli and Salmonella; the penicillinase (PenP) protein of B. licheniformis and B. cereus and S. aureus; pullulanase proteins of Klebsiella pneumoniae and Klebsiella aerogenese; E. coli lipoproteins lpp-28, Pal, RplA, RplB, OsmB, NlpB, and Orl17; chitobiase protein of V. harseyi; the β-1,4-endoglucanase protein of Pseudomonas solanacearum, the Pal and Pcp proteins of H. influenzae; the OprI protein of P. aeruginosa; the MalX and AmiA proteins of S. pneumoniae; the 34 kda antigen and TpmA protein of Treponema pallidum; the P37 protein of Mycoplasma hyorhinis; and the 17 kda antigen of Rickettsia rickettsii. It is to be understood, however, that the scope of the present invention is not to be limited to secretion signals of any particular lipoprotein or lipoproteins.

In one embodiment, the first DNA sequence may further include DNA which encodes all or a portion of the lipoprotein. Thus, in such an embodiment, the fusion protein which is expressed by the bacterium is a fusion protein of the secretion signal of the lipoprotein, all or a portion of the lipoprotein, and the heterologous protein or polypeptide or peptide.

The first and second DNA sequences are under the control of a suitable promoter. In one embodiment, the promoter may be the 19 kda antigen promoter or the 38 kda antigen promoter of M.tuberculosis if DNA encoding the secretion signal of one of these antigens is employed. Alternatively, the promoter may be a mycobacterial promoter other than the 19 kda and 38 kda M.tuberculosus antigen promoters, or a mycobacteriophage promoter.

Mycobacterial and mycobacteriophage promoters which may be employed include, but are not limited to, mycobacterial promoters

such as the BCG HSP60 and HSP70 promoters; the mycobactin promoter from M. tuberculosis and BCG; the mycobacterial 14 kda and 12 kda antigen promoters; the mycobacterial α-antigen promoter from M.tuberculosis or BCG; the MBP-70 promoter, the mycobacterial 45 kda antigen promoter from M.tuberculosis or BCG; the superoxide dismutase promoter; the mycobacterial asd promoter, and mycobacteriophage promoters such as the Bxb1, Bxb2, Bxb3, L1, L5, D29 and TM4 promoters. In one embodiment, the promoter is a mycobacterial heat shock protein promoter such as HSP60 or HSP70.

Example of expression vectors including the mycobacterial promoters and mycobacteriorphage promoters hereinabove described are further described in application Serial No. 642,017, filed January 16, 1991, which is a continuation of application Serial No. 552,828, filed July 16, 1990, now abandoned. The contents of application Serial No. 642,017 are hereby incorporated by reference.

In a preferred embodiment, the transcription initiation site, the ribosomal binding site, and the start codon, which provides for the initiation of the translation of mRNA, are each of mycobacterial origin. The stop codon, which stops translation of mRNA, thereby terminating synthesis of the heterologous protein, and the transcription termination site, may be of mycobacterial origin, or of other bacterial origin, or may be synthetic in nature, or such stop codon and transcription termination site may be those of the DNA encoding the heterologous protein or polypeptide.

Preferably, the mycobacterial promoter is a BCG promoter, and the mycobacterium is BCG.

Heterologous proteins or polypeptides which may be encoded by the second DNA sequence include, but are not limited to, antigens, anti-tumor agents, enzymes, lymphokines, pharmacologic

agents, immunopotentiators, and reporter molecules of interest in a diagnostic context.

Antigens which may be encoded include, but are not limited to, Mycobacterium leprae antigens; Mycobacterium tuberculosis antigens; Rickettsia antigens; Chlamydia antigens; Coxiella antigens; malaria sporozoite and merozoite proteins, such as the circumsporozoite protein from Plasmodium berghei sporozoites; diphtheria toxoids; tetanus toxoids; Clostridium antigens; Leishmania antigens; Salmonella antigens; E.coli antigens; Listeria antigens; Borrelia antigens, including the OspA and OspB antigens of Borrelia burgdorferi; Franciscella antigens; Yersinia antigens; Mycobacterium africanum antigens; Mycobacterium intracellulare antigens; Mycobacterium avium antigens; Treponema antigens; Schistosome antigens; Filaria antigens; Pertussis antigens; Staphylococcus antigens; Herpes virus antigens; influenza and parainfluenza virus antigens; measles virus antigens; Bordatella antigens; Hemophilus antigens; Streptococcus antigens, including the M protein of S.pyogenes and pneumococcus antigens such as Streptococcus pneumoniae antigens; mumps virus antigens; hepatitis virus antigens; Shigella antigens; Neisseria antigens; rabies antigens; polio virus antigens; Rift Valley Fever virus antigens; dengue virus antigens; measles virus antigens; rotavirus antigens; Human Immunodeficiency Virus (HIV) antigens, including the gag, pol, and env proteins; respiratory syncytial virus (RSV) antigens; snake venom antigens; human tumor antigens; and Vibrio cholera antigens. Enzymes which may be encoded include, but are not limited to, steroid enzymes.

In one embodiment, the second DNA sequence encodes at least one protein or polypeptide or fragment or derivative thereof which includes an epitope which is recognized by cytotoxic T lymphocytes induced by an HIV protein or fragment or derivative thereof. The at least one DNA sequence may encode an HIV protein or fragment or derivative thereof. HIV proteins or polypeptides

which may be encoded by the at least one DNA sequence includes but are not limited to, HIV-I-gp 120; HIV-I-gp 41; HIV-I-gp 160; HIV-I-pol; HIV-I-nef; HIV-I-tat; HIV-I-rev; HIV-I-vif; HIV-I-vpr; HIV-I-vpu; HIV-I-gag; HIV-2gp 120; HIV-2-gp 160; HIV-2-gp 41; HIV-2-gag; HIV-2-pol; HIV-2-nef; HIV-2-tat; HIV-2-rev; HIV-2-vif; HIV-2-vpr; HIV-2-vpu; and HIV-2-vpx.

Anti-tumor agents which may be encoded include, but are not limited to, interferon-α, interferon-β, or interferon-γ, and tumor necrosis factor, or TNF. Lymphokines which may be encoded include, but are not limited to, interleukins 1 through 8.

It is also contemplated that the heterologous protein or polypeptide may be a reporter molecule or selectable marker.

Reporter molecules which may be encoded include, but are not limited to, luciferase, B-galactosidase, B-glucuronidase, and catechol dehydrogenase.

Other peptides or proteins which may be encoded include, but are not limited to, those which encode for stress proteins, which can be administered to evoke an immune response or to induce tolerance in an autoimmune disease (e.g., rheumatoid arthritis).

Selectable markers which may be encoded include, but are not limited to, the β-galactosidase marker, the kanamycin resistance marker, the chloroamphenicol resistance marker, the neomycin resistance marker, and the hygromycin resistance marker, bacteriophage resistance markers, or genes encoding enzymes involved in the synthesis of nutritional elements, such as amino acids.

In accordance with one embodiment, the vector further includes a mycobacterial origin of replication.

In accordance with another embodiment, the vector may be a plasmid. The plasmid may be a non-shuttle plasmid, or may be a shuttle plasmid which further includes a bacterial origin of replication such as an E.coli origin of replication, a Bacillus origin of replication, a Staphylococcus origin of replication, a

Streptomyces origin of replication, or a streptococcal origin of replication. In one embodiment, the shuttle plasmid includes an E. coli origin of replication.

In accordance with yet another embodiment, the vector may further include a multiple cloning site, and the second DNA sequence encoding for the heterologous protein is inserted in the multiple cloning site.

In another embodiment, the expression vector may be, for example, a temperate shuttle phasmid or a bacterial-mycobacterial shuttle plasmid. Each of these vectors may be used to introduce the first DNA sequence encoding at least the secretion signal of a lipoprotein and a second DNA sequence encoding a protein or fragment thereof, or polypeptide or peptide heterologous to the mycobacterium which expresses the protein or fragment thereof, or polypeptide or peptide stably into mycobacteria, in which the DNA seqeunces may be expressed. When a shuttle phasmid, which replicates as a plasmid in bacteria and a phage in mycobacteria, is employed, integration of the phasmid, which includes the first DNA sequence encoding at least the secretion signal of a lipoprotein, and a second DNA sequence endoing a protein or fragment thereof, or polypeptide or peptide heterologous to the mycobacterium which expresses the protein or fragment thereof, or polypeptide or peptide, into the mycobacterial chromosome, occurs through site-specific integration. The DNA seqeunces are replicated as part of the chromosomal DNA. When a bacterial-mycobacterial shuttle plasmid is employed, the DNA sequences are stably maintained extrachormosomally in a plasmid. Expression of the DNA sequences occur extrachromosomally (e.g., episomally). For example, the DNA sequences are cloned into a shuttle plasmid and the plasmid is introduced into a mycobacterium such as those hereinabove described, wherein the plasmid replicates episomally. Examples of such shuttle phasmids and bacterial-mycobacterial shuttle plasmids are further

described in Application Serial No. 361,944, filed June 5, 1989, which is hereby incorporated by reference.

In addition to the first DNA sequence encoding at least the secretion signal of a lipoprotein and the second DNA sequence encoding a heterlogous protein or fragment thereof, or polypeptide or peptide, and the mycobacterial promoter for controlling expression of the DNA encoding the heterologous protein or polypeptide, the expression vector may, in one embodiment, further include a DNA sequence encoding bacteriophage integration into a mycobacterium chromosome. Bacteriophages from which the DNA sequence encoding bacteriophage integration into a mycobacterium chromosome may be derived include, but are not limited to, mycobacteriophages such as but not limited to, the L5, L1, Bxb1, and TM4 mycobacteriophages; the lambda phage of E. coli; the toxin phages of Corynebacteria; phages of Actinomycetes and Norcardia; the ϕC31 phage of Streptomyces; and the P22 phage of Salmonella. Preferably, the DNA sequence encodes mycobacteriophage integration into a mycobacterium chromosome. The DNA sequence which encodes bacteriophage integration into a mycobacterium chromosome may include DNA which encodes integrase, which is a protein that provides for integration of the vector into the mycobacterial chromosome. Preferably, the DNA sequence encoding mycobacterial phage integration also includes DNA which encodes an attP site.

The DNA encoding the attP site and the integrase provides for an integration event which is referred to as site-specific integration. DNA containing the attP site and the integrase gene is capable of integrating into a corresponding attB site of a mycobacterium chromosome.

It is to be understood that the exact DNA sequence encoding the attP site may vary among different phages, and that the exact DNA sequence encoding the attB site may vary among different mycobacteria.

Examples of DNA which is a phage DNA portion encoding bacteriophage integration into a mycobacterium chromosome are further described in Application Serial No. 869,330, filed April 15, 1992, which is a continuation-in-part of Application Serial No. 553,907, filed July 16, 1990, now abandoned. The contents of Application Serial No. 869,330 are incorporated by reference.

The vectors of the present invention may be employed to transform bacteria, and in particular, mycobacteria which include, but are not limited to, Mycobacterium bovis - BCG, M. smegmatis, M. avium, M. phlei, M. fortuitum, M. lufu, M. paratuberculosis, M. habana, M. scrofalaceum, M. intracellulare and M. vaccae; in particular, such vectors may be employed to transform BCG. The transformed mycobacteria thus express the heterologous protein, which, as hereinabove stated, may be an antigen, which induces an immune response, or a therapeutic agent. Thus, the transformed mycobacteria may be employed as part of a pharmaceutical composition, such as a vaccine and/or therapeutic agent, which includes the transformed mycobacteria, and an acceptable pharmaceutical carrier. Acceptable pharmaceutical carriers include, but are not limited, to mineral oil, alum, synthetic polymers, etc. Vehicles for vaccines and therapeutic agents are well known in the art and the selection of a suitable vehicle is deemed to be within the scope of those skilled in the art from the teachings contained herein. The selection of a suitable vehicle is also dependent upon the manner in which the vaccine or therapeutic agent is to be administered. The vaccine or therapeutic agent may be in the form of an injectable dose and may be administered intramuscularly, intravenously, orally, intradermally, or by subcutaneous administration.

The mycobacteria are administered in an effective amount. In general, the mycobacteria are administered in an amount of

from about 1 x 10^5 to about 1 x 10^{10} colony forming units (CEU's) per dose.

Other means for administering the vaccine or therapeutic agent should be apparent to those skilled in the art from the teachings herein; accordingly, the scope of the invention is not to be limited to a particular delivery form.

As hereinabove noted, the expression vectors of the present invention may contain DNA which encodes Borrelia antigen(s), including but not limited to surface proteins or antigens of Borrelia burgdorferi, the causative agent of Lyme disease. Thus, in accordance with an aspect of the present invention, there is provided a method of protecting an animal against Lyme disease which comprises administering to an animal mycobacteria transformed with DNA which includes at least one DNA sequence which encodes a protein or polypeptide which elicits antibodies against Borrelia burgdorferi. The mycobacteria are administered in an amount effective to protect an animal against Lyme disease. Such amounts may be those hereinabove described. In one embodiment, the at least one DNA sequence encodes a surface protein of Borrelia burgdorferi or a fragment or derivative thereof. Surface proteins of Borrelia burgedorferi which may be encoded by the at least one DNA sequence, include but are not limited to, Outer Surface Protein A and Outer Surface Protein B, sometimes hereafter referred to as OspA and OspB, respectively.

The transformed mycobacteria include those hereinabove described. In one embodiment, the mycobacteria are of the species M.bovis- BCG.

The at least one DNA sequence which encodes a protein or polypeptide which elicits antibodies against Borrelia burgdorferi, in a preferred embodiment, is contained in a mycobacterial expression vector. In one embodiment, the mycobacterial expression vector may include a DNA sequence encoding at least a secretion signal of a lipoprotein, such as

those hereinabove described, and wherein the mycobacterium expresses a chimeric fusion protein of the lipoprotein or lipoprotein segment (which may include the secretion signal) and the protein or polypeptide which elicits antibodies against Borrelia burgdorferi. Such an expression vector enables the protein or polypeptide which elicits antibodies against Borrelia burgdorferi, to be expressed on the surface of the mycobacterium, whereby the protein or polypeptide becomes more accessible.

It is also contemplated that, in another embodiment, the mycobacterial expression vector may contain DNA which encodes all or a portion of a mycobacterial excretion protein, as well as the DNA which encodes a protein or polypeptide which elicits antibodies against Borrelia burgdorferi. The mycobacterium expresses a fusion protein of the mycobacterial excretion protein or a portion thereof, and the protein or polypeptide which elicits antibodies against Borrelia burgdorferi. Such an expression vector enables the protein or polypeptide to be excreted from the mycobacterium. Examples of mycobacterial excretion proteins which may be encoded, include, but are not limited to, the α-antigen of M. tuberculosis and BCG.

The mycobacterial expression vector, in one embodiment, may include a promoter selected from the group consisting of mycobacterial promoters and mycobacteriophage promoters, such as those hereinabove described, and/or may include a DNA sequence encoding bacteriophage integration into a mycobacterium chromosome, also as hereinabove described.

In another embodiment, the mycobacterial expression vector may be a plasmid, such as a non-shuttle plasmid or a shuttle plasmid which further includes a bacterial origin of replication, also as hereinabove described.

It is also contemplated that the mycobacterial expression vector may be a temperate shuttle phasmid or a bacterial-mycobacterial shuttle plasmid as hereinabove described.

The transformed mycobacteria are employed as part of a composition for protecting an animal against Lyme disease. Such a composition includes the transformed mycobacteria, and an acceptable pharamaceutical carrier such as those hereinabove described.

The invention will now be further described with respect to the following examples; however, the scope of the present invention is not intended to be limited thereby.

Example 1

A. Construction of plasmids including mycobacterial promoter expression cassette.

1. Construction of pYUB125

Plasmid pAL5000, a plasmid which contains an origin of replication of M. fortuitum, and described in Labidi, et al., FEMS Microbiol. Lett., Vol. 30, pgs. 221-225 (1985) and in Gene, Vol. 71, pgs. 315-321 (1988), is subjected to a partial Sau 3A digest, and 5kb fragments are gel purified. A 5kb fragment is then ligated to Bam HI digested pIJ666 (an. E. coli vector containing an E. coli origin of replication and also carries neomycin-kanamycin resistance, as described in Kieser, et al., Gene, Vol. 65, pgs. 83-91 (1988) to form plasmid pYUB12. A schematic of the formation of plasmid pYUB12. A schematic of the formation of plasmid pYUB12 is shown in Figure 1. pYUB12 and pIJ666 were then transformed into M. smegmatis and BCG. Neomycin-resistant transformants that were only obtained by pYUB12 transformation confirmed that pAL5000 conferred autonomous replication to pIJ666 in M. smegmatis and BCG.

Shotgun mutagenesis by Snapper, et al (1988, hereinabove cited) indicated that no more than half of the pAL5000 plasmid was necessary to support plasmid replication in BCG. This segment presumably carried open reading frames ORF1 and ORF2, identified by Rauzier, et al., Gene, Vol. 71, pgs. 315-321

(1988), and also presumably carried a mycobacterial origin of replication. pYUB12 is then digested with HpaI and EcoRV, a 2586 bp carrying this region or segment pAL5000 is removed and ligated to PvuII digested pYUB8. Plasmid pYUB8 (a pBR322 derivative) includes an E. coli replicon and a kan^R (aph) gene. Ligation of the 2586 bp pYUB12 fragment to PvuII digested pYUB8 results in the formation of pYUB53, as depicted in Figure 2. Transformation of pYUB53 confirmed that the EcoRV-HpaI fragment, designated M.rep, was capable of supporting autonomous replication in BCG.

Plasmid pYUB53 was then digested with AatI, EcoRV, and PstI in order to remove the following restriction sites:

AatI 5707
EcoRI 5783
BamHI 5791
SalI 5797
PstI 5803
PstI 7252
SalI 7258
BamHI 7264
EcoRI 7273
ClaI 7298
HindIII 7304; and
EcoRV 7460

Fragment ends are then flushed with T4 DNA polymerase and religated to form plasmid pYUB125, construction of which is shown in Figure 3.

2. Elimination of superfluous vector DNA from pYUB125

792 bases of the tet gene, which had been inactivated by prior manipulations, was eliminated by a complete NarI digest, gel purification of the 6407 bp fragment, and ligation/recirculation, transformation of E. coli strain HB101, and selection of Kan^R transformants. The construction of resulting plasmid, pMV101, is schematically indicated in Figure

4, and the DNA sequence of pMV101, which includes markings of regions which will be deleted, and of mutations, as hereinafter described, is shown in Figure 5.

3. Elimination of undesirable restriction sites in aph (kan^R) gene.

To facilitate future manipulations, the HindIII and ClaI restriction sites in the aph gene were mutagenized simultaneously by polymerase chain reaction (PCR) mutagenesis according to the procedure described in Gene, Vol. 77 pgs. 57-59 (1989). The bases changed in the aph gene were at the third position of codons (wobble bases) within each restriction site and the base substitutions made were designed not to change the amino acid sequence of the encoded protein.

Separate PCR reactions of plasmid pMV101 with primers ClaMut-Kan + HindRMut-Kan and HindFMut-Kan + Bam-Kan were performed at 94°C (1 min.), 50°C (1 min.), and 72°C (1 min.) for 25 cycles. The PCR primers had the following base sequences:

ClaMut-Kan

CTT GTA TGG GAA GCC CC

HindRMut-Kan

GTG AGA ATG GCA AAA GAT TAT GCA TTT CTT TCC AG

HindFMut-Kan

GTC TGG AAA GAA ATG CAT AAT CTT TTG CCA TTC TCA CCG G

Bam-Kan

CGT AGA GGA TCC ACA GGA CG

The resulting PCR products were gel purified and mixed and a single PCR reaction without primers was performed at 94°C (1 min.), 72°C (1 min.) for 10 cycles. Primers ClaMut-Kan and Bam-Kan were added and PCR was resumed at 94°C (1 min.), 50°C (1 min.), and 72°C (2 min.) for 20 cylces. The resulting PCR product (Kan. mut) was digested with BamHI and gel purified. Plasmid pMV101 was digested with ClaI and cohesive ends were filled in by Klenow + dCTP + dGTP. Klenow was heat inactivated

and the digest was further digested with BamHI. The 5232 base pair fragment was gel purified and mixed with fragment Kan.mut and ligated. The ligation was transformed into E. coli strain HB101 and Kan^R colonies were screened for plasmids resistant to ClaI and HindIII digestion. Such plasmids were designated as pMV110, which is depicted in Figure 4.

4. Elimination of sequences not necessary for plasmid replication in mycobacteria.

Plasmid pMV110 was resected in separate constructions to yield plasmids pMV111 and pMV112. In one construction, pMV110 was digested with NarI and BalI, the ends were filled in, and a 5296 base pair fragment was ligated and recircularized to form pMV111. In another construct, pMV110 was digested with NdeI and SplI, the ends were filled in, and a 5763 base pair fragment was ligated and recircularized to form pMV112. Schematics of the constructions of pMV111 and pMV112 are shown in Figure 6. These constructions further eliminated superfluous E. coli vector sequences derived from pAL5000 not necessary for mycobacterial replication. Cloning was performed in E. coli. Plasmids pMV111 and pMV112 were tested for the ability to replicate in M. smegmatis. Because both plasmids replicated in M. smegmatis the deletions of each plasmid were combined to construct pMV113. (Figure 6).

To construct pMV113, pMV111 was digested with BamHI and EcoRI, and a 1071 bp fragment was isolated. pMV112 was digested with BamHI and EcoRI, and a 3570 bp fragment was isolated, and then ligated to the 1071 bp fragment obtained from pMV111 to form pMV113. These constructions thus defined the region of pAL5000 necessary for autonomous replication in mycobacteria as no larger than 1910 base paris.

5. Mutagenesis of restriction sites in mycobacterial replicon.

To facilitate further manipulations of the mycobacterial replicon, PCR mutagenesis was performed as above to eliminate the Sal I, EcoRI, and BglII sites located in the open reading frame known as ORF1 of pAL5000. PCR mutagenesis was performed at wobble bases within each restriction site and the base substitutions were designed not to change the amino acid sequence of the putative encoded ORF1 protein. The restriction sites were eliminated one at a time for testing in mycobacteria. It was possible to eliminate the SalI and EcoRI without altering replication in M. smegmatis. In one construction PCR mutagenesis was performed at EcoRI1071 of pMV113 with primers Eco Mut - M.rep and Bam-M.rep to form pMV117, which lacks the EcoRI1071 site. Primer Eco Mut - M.rep has the following sequence:

TCC GTG CAA CGA GTG TCC CGG A;

and Bam-M.rep has the following sequence:

CAC CCG TCC TGT GGA TCC TCT AC.

In another construction, PCR mutagenesis was performed at the SalI 1389 site with primer Sal Mut - M.rep and Bam-M.rep to form pMV119, which lacks the SalI 1389 site. Primer Sal Mut-M.rep has the following sequence:

TGG CGA CCG CAG TTA CTC AGG CCT.

pMV117 was then digested with ApaLI and BglII, and a 3360 bp fragment was isolated. pMV119 was digested with ApaLI and BglII, and a 1281 bp fragment was isolated and ligated to the 3360 bp fragment isolated from pMV117 to form pMV123. A schematic of the constructions of plasmids pMV117, pMV119, and pMV123 is shown in Figure 7. Elimination of the BglII site, however, either by PCR mutagenesis or Klenow fill in, eliminated plasmid replication in mycobacteria, thus suggesting that the BglII site is in proximity to, or within a sequence necessary for mycobacteria plasmid replication.

6. Construction of pMV200 series vectors.

To facilitate manipulations of all the components necessary for plasmid replication in E. coli and mycobacteria, (E. rep. and M. rep.) and selection of recombinants (Kan^R), cassettes of each component were constructed for simplified assembly in future vectrs and to include a multiple cloning site (MCS) containing unique restriction sites and transcription and translation terminators. The cassettes were constructed to allow directional cloning and assembly into a plasmid where all transcription is unidirectional.

Kan^R Cassette

A DNA cassette containing the aph (Kan^R) gene was constructed by PCR with primers $Kan^{5'}$ and $Kan^{3'}$. An SpeI site was added to the 5' end of the PCR primer Kan3', resulting in the formation of a PCR primer having the following sequence:

CTC GAC TAG TGA GGT CTG CCT CGT GAA G.

Bam HI + NheI sites were added to the 5' end of the primer Kan5', resulting in the formation of a PCR primer having the following sequence:

CAG AGG ATC CTT AGC TAG CCA CT GAC GTC GGG G.

PCR was performed at bases 3375 and 4585 of pMV123, and BamHI and NheI sites were added at base 3159, and an SpeI site was added at base 4585. Digestion with BamHI and SpeI, followed by purification resulted in a 1228/2443 Kan^R cassette bounded by BamHI and SpeI cohesive ends with the direction of transcription for the aph gene proceeding from BamHI to Spe I.

E. rep. cassette

A DNA cassette containing the ColEI replicon of pUC19 was constructed by PCR with primers E.rep/Spe and E.rep/Mlu. An SpeI site was added to the 5' end of PCR primer E.rep/Spe and an MluI site was added to the 5' end of PCR primer E.rep./Mlu. The resulting primers had the following sequences:

E.rep./Spe

CCA CTA GTT CCA CTG AGC GTC AGA CCC

E.rep./Mlu

GAC AAC GCG TTG CGC TCG GTC GTT CGG CTG.

PCR was performed at bases 713 and 1500 of pUC19, and an MluI site was added to base 713, and a SpeI site was added to base 1500. Digestion with MluI and SpeI, followed by purification resulted in an E.rep. cassette bounded by SpeI and MluI cohesive ends with the direction of transcription for RNA I and RNA II replication primers proceeding from SpeI to MluI.

M.rep. cassette

A DNA cassette containing sequences necessary for plasmid replication in mycobacteria was constructed by PCR of pMV123 with primers M.rep/Mlu and M.rep/Bam. An MluI site was added to the 5' end of PCR primer M.rep/Mlu. A BamHI site was added to the 5' end of PCR primer M/rep/Bam. The resulting PCR primers had the following base sequences:

M.rep./Mlu

CCA TAC GCG TGA GCC CAC CAG CTC CG

M.rep./Bam

CAC CCG TCC TGT GGA TCC TCT AC

PCR was performed at bases 134 and 2082 of pMV123. An MluI sited was added to base 2082. Digestion with BamHI and MluI, followed by gel purification resulted in a 1935 base pair DNA cassette bounded by MluI and BamHI cohesive ends with the direction of transcription for the pAL5000 ORF1 and ORF2 genes proceeding from MluI to Bam HI.

The Kan^R, E.rep, and M.rep PCR cassettes were then mixed in equimolar concentrations and ligated, and then transformed in E. coli strain HB101 for selection of Kan^R transformants. Colonies were screened for the presence of plasmids carrying all three cassettes after digestion with BamHI + MluI + SpeI and designated pMV200. An additional restriction site, NcoI, was eliminated from the M.rep cassette by digestion of pMV200 with NcoI, fill in with Klenow, and ligation and recircularization, resulting in the

formation of pMV201. A schematic of the formation of pMV200 from pMV123 and pUC19, and of pMV201 from pMV200, is shown in Figure 8. Plasmids pMV200 and pMV201 were transformed into M. smegmatis and BCG. Both plasmids yielded Kan^R transformants, thus indicating their ability to replicate in mycobacteria.

A synthetic multiple cloning sequence (MCS) (Figure 9) was then designed and synthesized to facilitate versatile molecular cloning and manipulations for foreign gene expressions in mycobacteria, and for integration into the mycobacterial chromosome. The synthetic MCS, shown in Figure 9, contains 16 restriction sites unique to pMV201 and includes a region carrying translation stop codons in each of three reading frames, and a T1 transcription terminator derived from E. coli rrnAB ribosomal RNA operon.

To insert the MCS cassette, pMV201 was digested with NarI and NheI, and the resulting fragment was gel purified. The MCS was digested with HinPI and NheI and, the resulting fragment was gel purified. The two fragments were then ligated to yield pMV204. A schematic of the construction of pMV204 is shown in Figure 10.

Plasmid pMV204 was then further manipulated to facilitate removal of the M.rep cassette in further constructions. pMV204 was digested with MluI, and an MluI - Not I linker was inserted into the MluI site between the M.rep and the E.rep to generate pMV206. A schematic of the construction of pMV206 from pMV204 is shown in Figure 11, and the DNA sequence of pMV206 is given in Figure 12.

7. Insertion of BCG HSP60 promoter sequence.

The published sequence of the BCG HSP60 gene (Thole, et al., Infect. and Immun., Vol. 55, pgs. 1466-1475 (June 1987)), and surrounding sequence permitted the construction of an HSP60 promoter fragment by PCR. The 251 bp HSP60 promoter fragment (Figure 13, and as published by Stover, et al. (1991)) was

amplified by PCR with primers including added XbaI and NheI sites. The PCR HSP60 fragment is then digested with XbaI and NheI, and is ligated into XbaI digested pMV206 to form pRB26 (Figure 14).

8. Insertion of DNA encoding the 19 kda M tuberculosis signal sequence and OspA gene into mycobacterial expression vector.

The sequence of the 19 kda M. tuberculosis gene is given in Ashbridge, et al., Nucleic Acids Research, Vol. 17, pg. 1249 (1989). The 19 kda antigen gene ribosomal binding site, start codon, and signal sequence from M. tuberculosis chromosomal DNA were amplified by PCR with nucleotide primers. The resulting 153 bp fragment (Figure 15) obtained by PCR includes added BglII (5') and BamHI: EcoRI sites (3'). This fragment contains the entire 5' region of the 19 kda gene up to the 27th codon with the exception of the promoter sequence. The PCR fragment is digested with BglII and EcoRI and ligated into BamHI-EcoRI digested pRB26 to form p2619S (Figure 16).

The gene encoding the OspA antigen is described in Bergstrom, et al., Molecular Microbiology, Vol. 3, No. 4, pgs. 479-486 (1989). The OspA gene sequence, excluding only the N-terminal 18 codons (encoding the secretion signal) was derived by PCR with added BamHI (5') and SalI (3') sites to provide a 780 bp OspA fragment. p2619S was digested with BamHI and SalI, and the 780bp PCR OspA fragment was digested with BamHI and SalI to generate cohesive ends and ligated to BamHI and SalI digested p2619S to form p2619::OspA. (Figure 17).

Example 2

Construction of mycobacterial vector including promoter and DNA encoding signal sequence of 19 kda M. tuberculosis antigen

Plasmid pMV206 was constructed as described in Example 1. The 19 kda M. tuberculosis antigen gene promoter, ribosomal binding site, start codon, and secretion signal was amplified by PCR with nucleotide primers. The PCR fragment includes added XbaI and BamHI sites. This sequence, shown in Figure 18, which is 286 bp in length, includes the entire published 5' region of the 19 kda gene up to the 27th codon. The PCR fragment was then digested with XbaI and BamHI, and ligated into XbaI and BamHI digested pMV206 to form p19PS (Figure 19). The 780 bp OspA PCR cassette, as described in Example 1, was digested with BamHI and SalI, and ligated to BamHI and SalI digested p19PS to form p19PS::OspA.

Example 3

Construction of mycobacterial expression vector with M. tuberculosis 38 kda antigen promoter and signal sequence and OspA gene

The gene sequence for the M. tuberculosis 38 kda antigen is given in Andersen, et al., Infection and Immunity, Vol. 57, No. 8, pgs. 2481-2488 (Aug. 1989). A DNA sequence encoding the 38 kda antigen promoter, ribosomal binding site, start codon, and secretion signal, obtained from M. tuberculosis chromosomal DNA, and containing the entire 5' sequence up to the 45th codon, was amplified by PCR with nucleotide primers. The resulting PCR fragment includes added XbaI and BamHI sites. The PCR fragment, 297 bp in length, and shown in Figure 20, was digested with XbaI and BamHI, and ligated into XbaI and BamHI digested pMV206 to form p38PS (Figure 21). The 780 bp OspA PCR cassette, as hereinabove described in Examples 1 and 2, is digested with BamHI and SalI and ligated into BamHI and SalI digested p38PS to form p38PS::OspA.

Example 4

Construction of mycobacterial expression vector with expression cassette based on BCG HSP60 and OspA gene

pMV206 was constructed as hereinabove described in Example 1.

The published sequence of the BCG HSP60 gene (Thole, et al, Infect. and Immun., Vol. 55, pgs. 1466-1475 (June 1987)), and surrounding sequence permitted the construction of a cassette carrying expression control sequences (i.e., promoter, ribosomal binding site, and translation initiation sequences as published in Stover, et al. (1991)) by PCR. The BCG HSP61 cassette (Figure 22) contains 375 bases 5' to the BCG HSP60 start codon, and 15 bases (5 codons) 3' to the start codon. PCR oligonucleotide primers were then synthesized. Primer Xba-HSP60, of the following sequence:

CAG ATC TAG ACG GTG ACC ACA ACG CGC C

was synthesized for the 5' end of the cassette, and primer Bam-HSP61, of the following sequence:

CTA GGG ATC CGC AAT TGT CTT GGC CAT TG

was synthesized for the 3' end of the cassette. The primers were used to amplify the cassette by PCR from BCG strain Pasteur chromosomal DNA. The addition of the Bam HI site at the 3' end of the cassette adds one codon (Asp) to the first six codons of the HSP60 gene.

Each of pMV206 and the PCR cassette HSP61 was digested with XbaI and BamHI. The PCR cassette was then inserted between the XbaI and BamHI sites of pMV206, then ligated to form plasmid pMV261. The construction of this plasmid is shown schematically in Figure 23.

The 780 bp OspA PCR cassette as herein ve described, was digested with BamHI and SalI, and ligated › BamHI and SalI digested pMV261 to form p261::OspA.

Example 5

A DNA cassette encoding the promoters and transcription start sites, as identified in Stover, et al. (1991), ribosome binding site, and start codon of the BCG HSP60 gene, was constructed by PCR. Such a cassette is the same as that of the BCG HSP61 cassette hereinabove described except that this cassette does not include the 15 bases (5 codons) 3' to the start codon. This cassette, which is 267 bp in length, and shown in Figure 24, includes added XbaI and NcoI sites, with a start codon included in the NcoI site. The cassette, after construction, was digested with XbaI and NcoI.

This cassette was placed into XbaI and NcoI digested pMV206 to form pMV251. (Figure 25). A full length OspA gene (including the signal sequence and as published in Bergstrom, et al. (1989)) was then derived by PCR as an NcoI-SalI restriction fragment. This fragment was then digested with NcoI and SalI, and ligated to NcoI and SalI digested pMV251 to form p251::OspA.

Example 6

pRB26 was constructed as described in Example 1. The 38 kda antigen gene ribosomal binding site, start codon, and secretion signal sequence was obtained from M. tuberculosis chromosomal DNA and amplified by PCR with nucleotide primers. The resulting fragment also includes added BglII-BamHI:EcoRI sites. The PCR fragment, 210 bp in length (Figure 26), is digested with BglII and EcoRI and ligated into BamHI and EcoRI digested pRB26 to form p2638S (Figure 27). p2638S is then digested with BamHI and SalI. The 780 bp OspA PCR fragment described in Example 1 is digested with BamHI and SalI and ligated to the BamHI and SalI digested p2638S to form p2638::OspA.

Example 7

This example describes the formation of p3638::OspA, which includes sequences encoding bacteriophage integration into a mycobacterium chromosome, DNA encoding the secretion signal of the 38 kda M. tuberculosis antigen, as well as the OspA gene.

pMV206 was constructed as hereinabove described in Example 1.

Plasmid pMH9.4, which includes the mycobacteriophage L5 attP site, and the L5 integrase gene, was employed in providing the L5 integration sequences to a BCG expression vector. The construction of pMH9.4, as well as its integration into M. smegmatis and BCG, is described below in sections (i) through (vi).

(i) Identification of the DNA sequences of the attachment sites, attB, attL, and attR, of M.smegmatis.

Using standard technologies, a lambda EMBL3 library was constructed using chromosomal DNA prepared from $mc^2$61 (a strain of M. smegmatis which includes an M. smegmatis chromosome into which has been integrated the genome of mycobacterial phage L5) and digested with Bam HI. Phage L5 contains DNA having restriction sites identical to those of phage L1 (Snapper, et al. 1988), except that L5 is able to replicate at 42°C and phage L1 is incapable of such growth. This library was then probed with a 6.7 kb DNA fragment isolated from the L5 genome that had been previously identified as carrying the attP sequence (Snapper, et al 1988). One of the positive clones was plaque purified, DNA prepared, and a 1.1 kb Sal I fragment (containing the AttL sequence) sub-cloned into sequencing vector pUC119. The DNA sequence of this fragment was determined using a shotgun approach coupled with Sanger sequencing. By isolating and sequencing the attL junction site and comparing this to the DNA sequence of L5 that was available, a region was determined where the two sequences aligned but with a specific discontinuity present. The discontinuity represents one side of a core sequence, which is

identical in AttP, attB, and attL. The region containing the recombinational crossover point is shown in Figure 28.

The attL DNA (1.1 kb Sal I fragment) was used as a probe to hybridize to a Southern blot of Bam HI digested $mc^2$6 DNA, which is a strain of M. smegmatis which includes an M. smegmatis chromosome without any phage integration (Jacobs, et al, 1987, hereinabove cited.). A single band of approximately 6.4 kb was detected corresponding to the attB sequence of M. smegmatis. This same attL probe was used to screen a cosmid library of $mc^2$6 (provided by Dr. Bill Jacobs of the Albert Einstein College of Medicine of Yeshiva University), and a number of positive cosmid clones were identified. DNA was prepared from these clones, and a 1.9 kb Sal I fragment (containing the attB site) that hybridizes to the attL probe was subcloned into pUC119 for sequencing and further analysis. The DNA sequence containing the core sequence was determined and is shown in Figure 28. The core sequence, which is identical in attP, attB and attL, has a length of 43bp.

The $mc^2$61 lambda EMBL3 library was then probed with the 1.9kb SalI fragment containing the attB site. Positive plaques were identified, DNA was prepared, and analyzed by restriction analysis and Southern blots. Lambda clones were identified that contained a 3.2kb Bam HI fragment containing the putative attR site. The 3.2kb Bam HI fragment was purified and cloned into pUC119 for sequencing and further analysis.

(ii) Determination of attP-integrase region of L5 genome.

Concurrent with the above procedures, a significant portion of the DNA sequence of L5 had been determined and represented in several "contigs" or islands of DNA sequence. Sequences of the 6.7kb Bam HI fragment hereinabove described were determined by (a) analysis of the location of Bam HI sites in the contigs of the DNA of L5, and (b) by determining a short stretch

of DNA sequence from around the Bam HI sites of plasmid pJR-1 (Figure 33), which carries the 6.7kb Bam HI fragment of L5.

A segment of DNA sequence was located that represented the 6.7kb Bam HI fragment of phage L5. Studies of other phages have shown that the integrase genes are often located close to the attP site. It was thus determined that the L5 integrase (int) gene should lie either within the 6.7kb Bam HI fragment or in a DNA sequence on either side of it. The DNA sequence in the regions was then analyzed by translating it into all six possible reading frames and searching these amino acid sequences for similarity to the family of integrase related proteins, and through computer-assisted analysis of the DNA sequence. As shown in Figure 29, there are shown two domains of reasonably good conservation among L5 integrase and other integrases, and three amino acid residues that are absolutely conserved in domain 2. (See Yagil, et al., J. Mol. Biol., Vol. 207, pgs. 695-717 (1989), and Poyart-Salmeron, et al., J. EMBO., Vol. 8, pgs. 2425-2433 (1989)). A region was identified, and analysis of the corresponding DNA sequence showed a reading frame that could encode for a protein of approximately 333 amino acids. These observations identified the putative int gene.

The location of the int gene was not within the 6.7kb Bam HI fragment; however, it was very close to it with one of the Bam HI sites (that defines the 6.7kb Bam HI fragment) less than 100 bp upstream of the start of the gene. Analysis of the Bam HI sites showed that the int gene lay within a 1.9kb Bam HI fragment located adjacent to the 6.7kb Bam HI fragment. This 1.9kb Bam HI fragment was cloned by purification of the fragment from a Bam HI digest of L5 DNA and cloning into pUC 119, to generate pMH1 (Figure 34).

From a combination of the above approaches, a schematic of the organization of the attP-int region of L5 was constructed

(Figure 30), and the gene sequence of the attP-int region is given in Figure 31.

(iii) Construction of pMH5.

The 6.7kb Bam HI fragment of mycobacteriophage L5, which contains the attP site, as hereinabove described, was cloned into the Bam HI site of pUC 119 (Figure 32). This was achieved by purifying the 6.7kb Bam HI fragment from a Bam HI digest of L5 DNA separated by agarose gel electrophoresis and ligating with Bam HI cut pUC 119. DNA was prepared from candidate recombinants and characterized by restriction enzyme analysis and gel electrophoresis. A recombinant was identified that contained the 6.7kb Bam HI fragment of L5 cloned into pUC 119. This plasmid was named pJR-1, as shown in Figure 33.

Analysis of DNA sequence data from a project to sequence L5 showed that a 1.9kb Bam HI fragment adjacent to the 6.7kb Bam HI fragment hereinabove described contained the integrase gene.

A plasmid containing a 1.9kb Bam HI fragment containing the DNA encoding for the integrase cloned into the Bam HI site of pUC 119 was constructed. The 1.9kb fragment was purified from a Bam HI digest of L5 DNA and cloned into the Bam HI site of pUC 119. Construction of the recombinant was determined by restriction analysis and gel electrophoresis. This plasmid was called pMH1, the construction of which is shown schematically in Figure 34.

pJR-1 was then modified by digestion with EcoRI and SnaBI (both are unique cloning sites), between which is a Bam HI site. The Eco RI-Sna BI fragment, including the Bam HI site was excised, and the plasmid was religated to form plasmid of pMH2, which contains on Bam HI site compared to two Bam HI sites contained in pJR-1. A schematic of the construction of pMH2 is shown in Figure 35.

The 1.9kb Bam HI fragment, which includes the integrase gene, was purified from a Bam HI digest of pMH1 and ligated to

Bam HI digested pMH2. Recombinants were identified as above and the orientation of the 1.9kb fragment determined. A plasmid called pMH4 was thus constructed (Figure 36) in which the region from the Sna BI site (upstream of attP) through to the Bam HI site (downstream of the integrase gene) was identical to that in L5.

pMH4 was digested with HindIII (unique site) and was ligated to a 1kb HindIII fragment purified from pKD43 (supplied by Keith Darbyshire of the Nigel Gindley Laboratory) that contains the gene determining resistance to kanamycin. Recombinants were identified and characterized as above. This plasmid is called pMH5. A schematic of the construction of pMH5 is shown in Figure 37.

(iv) Integration of pMH5 into attB of M. smegmatis.

Plasmids pYUB12 (a gift from Dr. Bill Jacobs, a schematic of the formation of which is shown in Figure 1), pMD01 (Figure 38), and pMH5 were electroporated, with four different concentrations of plasmid DNA over a 1,000-fold range, into M. smegmatis strain mc^2155, a strain which is able to support plasmid replication. In sections (iv) through (vi), all electroporation procedures of M. smegmatis, or of BCG, were carried out as follows:

Cultures of organism were grown in Middlebrook 7H9 media, as described by Snapper, et al. (1988), harvested by centrifugation, washed three times with cold 10% glycerol, and resuspended at approximately a 100 x concentration of cells.

1 µl of DNA was added to 100 µl of cells in an ice-cold cuvette and pulsed in a Bio-Rad Gene Pulser, and given a single pulse at 1.25 kv at 25 µF. 1 ml of broth was added the cells incubated for 1 hr. at 37°C for expression of the antibiotic-resistant marker. Cells were then concentrated and plated out on Middlebrook or tryptic soy media containing 15 µg/ml kanamycin. Colonies were observed after 3 to 5 days incubation at 37°C.

Each of pYUB12, pMD01, and pMH5 carries kanamycin resistance. Plasmid pYUB12 carries an origin of DNA replication, while pMD01 lacks a mycobacterial origin of replication. Plasmid pMH5 does not carry a mycobacterial origin of replication, but carries a 2kb region of phage L5 which contains the attP site and the integrase gene (Figure 31). The number of transformants were linear with DNA concentration. Plasmid pYUB12 gives a large number of transformants (2×10^5 per µg DNA) in mc^2155, while pMH5 gives 6×10^4 transformants per µg DNA, and pMD01 gives no transformants.

The above experiment was then repeated by electroporating the plasmids pYUB12, pMD01, and pMH5 into M. smegmatis strain mc^26, which does not support plasmid replication. No transformants in mc^26 were obtained from pYUB12 or pMD01, while pMH5 gave approximately 10^4 kanamycin resistant transformants in mc^26 per µg of DNA, thus indicating integration of pMH5 into the mc^26 chromosome.

DNA from six independent pMH5 transformants (four in mc^2 155 and two in mc^26) was prepared. These DNA's (along with DNA from both mc^2155 itself, and mc^2155 carrying the plasmid pYUB12) were digested with a restriction enzyme, and analyzed by Southern blot and hybridization with the M. smegmatis 1.9kb attB probe hereinabove described. As shown in Figure 39, all six transformants have integrated into the attB site, resulting in the production of two new DNA fragments with different mobilities. If pMH5 did not integrate into the attB site, it would be expected that a single band, corresponding to the attB site in the mc^2155 control, would be obtained.

(v) Construction of pMH9.2 and pMH9.4

pUC119 was digested with HindIII, and a 1kb HindIII fragment, containing a kanamycin resistance gene, purified from pKD43, was ligated to the HindIII digested pUC119 to form pMH8 (Figure 40). A 2kb SalI fragment (bp 3226-5310), which carries

the attP and integrase gene from SalI digested pMH5, was purified and inserted in both orientations relative to the vector backbone of SalI digested pMH8 to form plasmids pMH9.2 and pMH9.4 (Figures 41 and 42).

M. smegmatis strain mc^2155 cells carrying, as a result of electroporation, plasmid pYUB12, pMH9.2 or pMH9.4, or strain mc^26 cells carrying plasmid pMH5, as a result of electroporation as hereinabove described, were grown to saturation in broth with kanamycin. Cultures were then diluted 1:100 into broth without kanamycin and grown to saturation. Two further cycles of dilution and growth were done, corresponding to about 20 generations of bacterial growth. Cultures were plated out to single colonies on non-selective plates, and approximately 100 of these colonies were patch plated onto both non-selective and selective plates. The % of colonies that were sensitive to kanamycin, thus corresponding to the percentage of cells which lost the plasmid, is given below in Table I.

Table I

	% loss
pYUB12 (mc^2155)	35
pMH5 (mc^26)	17
pMH9.2 (mc^2155)	3
pMH9.4 (mc^2155)	0

(vi) Transformation of BCG with pMH9.4

The 1.9 kb Sal I fragment, which includes the M. smegmatis attB site as hereinabove described was cloned into pUC119, and the plasmid generated was named pMH-12. (Figure 43).

Gel purified Sal I 1.9kb M. smegmatis fragment containing attB (isolated from pMH-12) was used to probe a Southern transfer of Bam HI digested mycobacterial DNA's, including BCG substrain Pasteur, shown in Figure 44. This demonstrated that there is one Bam HI fragment of BCG that strongly hybridizes to the M. smegmatis attB probe and three hybridize weakly. The strongest

hybridizing band is the fastest moving band (approximately 1.9 kb).

The same probe as above was used to probe a BCG cosmid library (provided by Dr. Bill Jacobs) and positive clones were identified. DNA was prepared from several positive clones and analyzed by restriction analysis and Southern blotting. The 1.9 kb Bam HI fragment (corresponding to the strongly hybridizing band in the Southern blot was identified, gel purified from the cosmid DNA and cloned into pUC119. The resulting plasmid was named pMH-15. (Figure 45).

Plasmid pMH-5 and pMH9.4 were electroporated into BCG Pasteur. It was observed that pMH9.4 transforms BCG with high efficiency (approximately 10^4 transformants/μg DNA), while pMH-5 transforms BCG at low efficiency (1-10 transformants/μg DNA). DNA was prepared from BCG transformants and analyzed by Bam HI restriction and Southern blot analysis, probing with gel purified 1.9kb Bam HI BCG attB fragment from pMH-15. These data are shown in Figure 41 and show that integration of both pMH5 and pMH9.4 is specific to the BCG attB site (ie. the strongly cross-hybridizing fragment in BCG). This is illustrated by the loss of the 1.9kb Bam HI fragment from the transformants and the appearance of two new bands representing attL and attR junction fragments. Figure 46 shows just one of the pMH5/BCG transformants, although all of the four that were analyzed show that one of the bands (the largest) is smaller than expected (and different in each of the transformants), indicating that the transformation effiency of pMH-5 is low in BCG. In contrast, the four pMH9.4 transformants are identical to each other (Figure 46) and give attR and attL junction fragments of the predicted sizes.

Plasmid pMV206 was digested with NotI to remove the mycobacterial replicon. The resulting 2209 bp fragment, which includes the aph (Kan^R) gene, the E. coli replicon and the multiple cloning site, was ligated and recircularized to form

pMV205, the construction of which is schematically depicted in Figure 11.

PCR with primers XbaI-Att/Int and NheI-Att/Int was then performed on a Sal I fragment from pMH9.4, which contains the attP site and the L5 integrase gene. The resulting cassette was then digested with XbaI and NheI and a 1789 bp fragment was gel purified. pMV205 was then digested with NheI, and the resulting fragment was ligated to the 1989 bp fragment obtained from pMH9.4 to form pMV306. A schematic of the construction of pMV306 is shown in Figure 47.

p2638::OspA (from Example 6) and pMV306 were each digested with XbaI and SalI. The XbaI-SalI fragment of p2638:OspA, which contains the HSP60 promoter, 38 kda secretion signal sequence, and OspA antigen sequence, was ligated into XbaI and SalI digested pMV306 to form p3638::OspA.

Example 8

pRB26 was constructed as described in Example 1. The 32 kda α-antigen gene of M. tuberculosis or BCG (Matsuo, et al., J. Bacteriol, Vol. 170, No. 9, pgs 3847-3854 (Sept. 1988); Borremans, et al., Infect. and Immun., Vol. 57, No. 10, pgs. 3123-3130 (Oct. 1989)) was obtained from BCG chromosomal DNA and amplified by PCR using primers including added BglII-BamHI:EcoRI sites. The PCR fragment, 420 bp in length (Figure 48), was digested with BglII and EcoRI, and ligated into BamHI and EcoRI digested pRB26 to form pAB261 (Figure 49), which contains the entire α-antigen gene. pAB261 was then digested with BamHI and SalI, and the 780bp PCR OspA cassette hereinabove described in Example 1, was also digested with BamHI and SalI, and was ligated to BamHI and SalI digested pAB261 to form pAB261::OspA.

Example 9

Plasmid pMV206 was constructed as hereinabove described in Example 1.

A partial sequence of the 5' region of the BCG HSP70 gene (which encodes the BCG HSP70 heat shock protein, also known as the 70 kda antigen) obtained by Dr. Raju Lathigra (Medical Research Council, London) permitted the construction of a cassette carrying the promoter sequence. The HSP70 promoter was amplified by PCR with primers including Xba and NheI sites. The HSP70 promoter PCR fragment, 121 bp in length (Figure 50), was digested with XbaI and NheI, and ligated to XbaI digested pMV206 to form pRB27. (Figure 51.) The 32 kda α-antigen gene of BCG was obtained from BCG chromosomal DNA as described in Example 8, and amplified by PCR using primers including added BglII-BamHI:EcoRI sites. The PCR fragment was digested with BglII and EcoRI, and ligated into BamHI and EcoRI digested pRB27 to form pAB271 (Figure 52), which contains the entire α-antigen gene. pAB271 was then digested with BamHI and SalI, and the 780bp PCR OspA cassette hereinabove described in Example 1, was also digested with BamHI and SalI, and was ligated to BamHI and SalI digested pAB271 to form pAB271::OspA.

Example 10

Vectors p19PS::OspA, p38PS::OspA, pMV261::OspA, and pMV251::OspA were transformed into BCG. The transformed BCG cells were cultured, and the cells were then sedimented from the cultures. The cells were then suspended in phosphate buffered saline (PBS), and cell suspensions were normalized to equivalent densities. The cells were disrupted by sonication, the cell envelopes were sedimented, and the supernatant (a Cytosol-enriched fraction) was saved. The cell envelopes were resuspended in PBS, and membranes were solubilized at 4°C by the addition of Triton X-114 to 2% (vol./vol.). Insoluble material (a cell wall-enriched fraction) was sedimented, and the

supernatant (membrane-enriched fraction) was removed. Triton X-114 was added to the Cytosol-enriched fraction. After brief warming of the Triton X-114 solutions at 37°C, separation of aqueous and detergent phases was achieved by a short centrifugation. These two phases were back-extracted three times, and proteins in representative samples were precipitated by the addition of acetone. A portion of each culture supernatant was concentrated by an ultrafiltration device (Centricon-30, Amicon). Samples representing culture volume equivalents were processed by SDS-PAGE, transferred to nitrocellulose, and Western blotted with anti-OspA monoclonal antibody (Mab) H5332. (Howe, et al., Infect. and Immun., Vol. 54, No. 1, pgs. 207-212 (Oct. 1986)). Filter-bound antibody was visualized with an enhanced chemiluminescence. system (Amersham). As shown in Figure 53, Lane 1 is a molecular weight standard (Rainbow Markers, Amersham); lane 2 is a whole cell sonicate fraction; lane 3 is Triton X-114 insoluble material; lane 4 is the aqueous phase membrane fraction; lane 5 is the detergent phase membrane fraction; lane 6 is the aqueous phase Cytosol fraction; lane 7 is the detergent phase Cytosol fraction; and lane 8 is a concentrated culture medium.

As can be seen from Figure 53, recombinant chimeric OspA fusion proteins expressed from the expression vectors p19PS::OspA and p38PS::OspA were found to be localized predominantly in the Triton X-114 phase from the membrane fractions, thus suggesting that these recombinant OspA proteins were fused to the mycobacterial 19 kda and 38 kda secretion signals, which directed secretion and post-translational processing by fatty acylation at an N-terminal cysteine. OspA expressed with its native lipoprotein signal peptide by pMV251::OspA was found to be localized in detergent soluble BCG membrane fractions although additional OspA was also found in BCG cytoplasmic aqueous fractions, thus suggesting that the OspA signal was not as

efficiently processed in BCG as were the 19 kda and 38 kda signal sequences. Recombinant OspA expressed by pMV261::OspA, wherein OspA was not fused to a lipoprotein signal, was found to be localized only in aqueous cytoplasmic fractions.

Example 11

BCG cells were transformed with either pAB261::OspA, pAB271::OspA, or pMV261::OspA and cultured. Portions of BCG culture supernatants were depleted of bovine serum albumin (BSA), a component of the medium, by adsorption with Affi-gel Blue (Bio Rad). BCG cell pellets from the cultures were suspensed in PBS and sonicated. Adsorbed or unadsorbed supernatants were concentrated (Centricon 30) and then diluted to the same relative concentration, on a culture volume basis, as the lysed cells. Samples were used for SDS-PAGE and subsequent immunoblotting with anti-OspA (Mab H 5332), anti-Hsp70 (Mab IT-41, WHO mycobacterial monoclonal antibody bank), or anti-Hsp60 (Mab IT-13, WHO mycobacterial monoclonal antibody bank). As shown in Figure 54, lane M.W. Std. is a molecular weight standard, lanes W are whole cell lysates, lanes S are culture supernatants (unadsorbed), and lanes A are adsorbed supernatants. As shown in Figure 54, it was determined that fusion of the OspA gene, without the secretion signal, to the complete α-antigen gene resulted in high level expression, and a substantial fraction of the resulting recombinant α-antigen-OspA fusion protein was found to be excreted into the culture media. The absence of detectable quantities of cytoplasmic proteins (Hsp60 and Hsp70) in the supernatant indicated that cell lysis was minimal, and that the recombinant α-antigen::OspA fusion protein was specifically targeted to be secreted and is not simply found in culture supernatants due to autolysis.

Example 12

BCG organisms (Pasteur strain) were transformed with one of the following vectors:

pMV261::OspA

pMV251::OspA

p2619::OspA

p19PS::OspA

p38PS::OspA

p3638::OspA

pAB261::OspA

As a negative control, pMV261/LZ was used to transform a control group of organisms. pMV261/LZ was constructed by cloning a BamHI restriction fragment carrying the E.coli lacZ gene (which encodes ß-galactosidase) into the BamHI site of Bam HI digested pMV261.

The transformed BCG colonies were isolated by selection for kamamycin resistance and expanded in liquid media culture for further analysis. Recombinant BCG samples representing culture volume equivalents were processed by SDS-PAGE, transferred to nitrocellulose, and Western blotted with anti-OspA Mab H5332. Positive controls employed were a processed sample of B.burgdorferi strain B31, and samples of OspA antigens in concentrations of 500 µg/ml, 100 µg/ml. and 20 µg/ml. The filter-bound antibody was visualized with an enhanced chemilumineseuce system (Amersham). As shown in Figure 55, OspA was expressed by BCG transformed with vectors including the OspA gene. Figure 55 also shows the expression of a fusion protein of OspA and a mycobacterial secretion signal by BCG transofmred with p2619::OspA, p19PS::OspA, p38PS::OspA, or p3638::OspA.

Example 13

BCG organisms were transformed with pMV261::OspA, and the transformed organisms were cultured. Twenty-four different

strains of mice, with five mice representing each strain, were immunized with a single dose of 1 x 10^6 CFU of BCG transformed with pMV261::OspA (post freeze titer of 42%) intraperitoneally. The mice were bled every four weeks for 16 weeks, and also at 19 weeks. Sera were analyzed by ELISA on whole cells of Borrelia burgdorferi and BCG lysate coated on wells. The reaction was developed with peroxidase conjugated anti-mouse immunoglobulin and substrate. Color development was read as absorbance at 405nm. Positive sera had optical density (O.D.) values at three standard deviations above the mean of the prebleed sera. At 17 weeks, the mice were given a booster intraperitoneal injection of 1 x 10^6 CFU of BCG transformed with pMV261::OspA.

As shown in Figures 56 and 57, the following strains:

A/HeJ
A/J
AKR/J
BALB/cByJ
CBA/J
C3H/HeJ
SJL/J
LP/J
129/J
CE/J
B10.BR/SgSnJ
D4 Swiss Webster
SenCar
FVB

showed an immune response after a single immunization, and the following strains:

A/HeJ
A/J
C3H/HeJ
129/J

CE/J

B10.BR/SgSnJ

D4 Swiss Webster

had responded with significantly high levels of antibody against Borrelia burgdorferi.

Example 14

BCG organisms transformed with either pMV261::OspA, pAB261::OspA, p19PS::OspA, or pMV251::OspA, plus non-recombinant BCG Pasteur organisms were subjected to cell fractionation and Triton X-114 detergent phase partitioning analysis (Bordier, et al; J. Biol, Chem., Vol. 256, pg. 1604 (1981); Radolf, et al., Infect and Immun., Vol. 56, pg. 490 (1988)) to determine if expression of OspA genes in the vectors hereinabove described resulted in export and lipid acylation of recombinant OspA protein.

Recombinant BCG cells wee sedimented from BCG cultures, suspended in phosphate buffered saline (PBS), and cell suspensions were adjusted to equivalent densities. Cells were disrupted by sonication and mebranes were solubilized at 4°C by the addition of Triton X-114 to 2% (vol./vol.). Insoluble material (cell wall enriched fraction) was centrifuged, and the supernatant was subjected to detergent phase partitioning. After briefly warming (37°C) the Triton X-114 solutions, separation of aqueous and detergent phases was achieved by a short centrifuguation . The two phases were back-extracted three times, and proteins in representative samples were precipitated by the addition of acetone. A portion of each culture supernatant was concentrated by ultrafiltration. Samples representing 5-fold concentrated culture volume equivalents were processed by SDS-PAGE, transferred to nitrocellulose and blotted with anti-OspA MAb H5332. (Figure 58). Similar fractions from non-recombinant BCG were blotted with appropriate monoclonal

antibodies specific for the BCG or M.tuberculosis Hsp60 protein (IT13), α antigen (HYT27), or M.tuberculosis 19kda antigen (HYT6) to determine the cellular location of the native fusion partners. As shown in Figure 58, lane W is a whole cell sonicate fraction; lane I is a Triton X-114 insoluble cell wall enriched fraction; lane A is a cytosol-enriched aqueous fraction; lane D is a detergent phase (membrane-enriched) fraction and lane M is a 5-fold concentrated culture medium fraction.

As shown in Figure 58, the OspA gene product encoded by pMV261::OspA was found excessively in the aqueous cytosolic fraction (lane A) and correlated with the exclusive cytoplasmic location of HSP60. The α-antigen-OspA gene product expressed by pAB261::OspA and the native BCG α-antigen were found in the insoluble cell wall enriched fraction (lane I), aqueous cytosolic fraction (lane A), and media fraction (lane M), but not in the detergent soluble lipoprotein-enriched fraction (lane D). The presence of the α-antigen in the recombinant BCG culture media was not due to recombinant BCG autolysis, as HSP60 was not found in the culture media. Compared to the native BCG α-antigen, a substantially smaller fraction of the fusion protein expressed by pAB261::OspA was secreted into the media, while a larger portion was found in the cell wall enriched insoluble fraction. This suggests that fusion to the α-antigen could also direct foreign antigens to the cell wall. Substitution of the M. tuberculosis 19kda antigen signal peptide for the OspA signal peptide resulted in expression of a chimeric OspA protein that was located almost exclusively in the detergent soluble fraction. This finding indicated that fusion of the M. tuberculosis 19kda antigen signal peptide to OspA did direct efficient expression and export of the OspA protein to the membrane of BCG. This result was in contrast to the product expressed by organisms transformed with pMV251::OspA, where most of OspA was found in the aqueous

fraction, which may have been due to inefficient processing of the native Borrelia signal peptide.

Example 15

The recombinant BCG organisms of Example 14 were analyzed by flow cytometry to determine if the recombinant OspA gene products were accessible on the surface of recombinant BCG to anti-OspA antibody.

Approximately 2 x 10^8 recombinant BCG organisms grown in Dubos media supplemented with albumin-dextrose complex and 0.05% Tween 80 were harvested by centrifugation. The pelleted recombinant BCG organisms were washed with 10 ml. of phosphate buffered saline (pH 7.4) containing 0.05% Tween 80 (PBS-T80), resuspended in 5 ml. PBS-T80, and fixed for 10 minutes in 2% paraformaldehyde. Fixed recombinant BCG organisms were pelleted and washed twice with 5 ml. PBS-T80, and then resuspended in 1 ml. of PBS-T80. Polyclonal rabbit sera specific for OspA (BCG-adsorbed) was added to the fixed recombinant BCG cell suspension to a final dilution of 1:200 and incubated for 30 minutes at room temperature and 30 minutes on ice. The suspension was then pellted by centrifugation, washed twice with 0.5 ml. PBS-T80 and resuspended in 1 ml. PBS-T80. Goat anti-rabbit FITC-conjugated secondary antibody was added to a final dilution of 1:50 and incubated for 30 minutes on ice. The recombinant BCG-secondary antibody suspension was pelleted by centrifugation, washed twice with 1 ml. PBS-T80 and resuspended in 2 ml. PBS-T80. Labeled recombinant BCG were mildly sonicated to disperse clumped cells and dilutions were analyzed by flow cytometry on an FACS scan (Becton-Dickinson). Recombinant BCG containing the designated plasmids and expressing the designated chimeric OspA gene products are compared to non-recombinant BCG. (Figure 59).

As shown in Figure 59, recombinant BCG organisms expressing OspA from plasmids p19PS:OspA, pMV251::OspA, and pAB261::OspA, all demonstrated increased surface fluorescence with anti-OspA sera when compared with non-recombinant BCG or recombinant BCG expressing OspA from plasmid pMV261::OspA. The relative surface fluorescence exhibited by expression of OspA from organisms transformed with pMV251::OspA was less than that observed for organisms transformed with p19PS::OspA, and was in agreement with the fractionation analysis of Example 14. The recombinant BCG expressing OspA from pAB261::OspA also exhibited surface flourescence, thus confirming that the α-antigen-OspA fusion protein found in the Triton insoluble fraction (Example 14) was cell wall associated and not derived from insoluble inclusion bodies. Therefore, it was possible to export OspA to the surface of BCG as a membrane-associated lipoprotein by fusion to the M. tuberculosis 19kda antigen signal sequence, or as a secreted and cell wall associated protein by fusion to the α-antigen.

Example 16

C3H/He, BALB/C, and Swiss Webster mice were immunized with 10^6 colony forming units of BCG organisms transformed with pMV261::OspA, pMV251::OspA, p19PS::OspA, pAB261::OspA, or of non-recombinant BCG Pasteur. The mice were given a booster of the identical dose at 16 weeks. As shown in Figure 60, all three mouse strains immunized with BCG transformed with pMV251::OspA or p19PS::OspA exhibited strong OspA-specific antibody responses within 4 to 8 weeks after a single immunization as measured by ELISA to whole Borrelia organisms or purified OspA. Particularly striking were the anti-OspA responses elicited by a single dose of BCG organisms transformed with either pMV251::OspA or p19PS::OspA; in the low responder Swiss Webster strain; the same strain of mice immunized with BCG transformed with pMV261::OspA or pAB261::OspA did not mount anti-OspA responses even after boosting. Peak anti-OspA antibody titers exceeding $1:10^5$ in

BALB/C and C3H/He mice, and $1:10^4$ in Swiss Webster mice were elicited by boosting with BCG transformed with pMV251::OspA or p19PS::OspA, and these responses were 100 to 1,000-fold higher than the responses induced with BCG transformed with pMV261::OspA or pAB261::OspA.

Example 17

Immune sera from the immunized C3H/He and BALB/C mice of Example 16 were analyzed for their ability to inhibit growth of the non-pathogenic B31 laboratory strain of B. burdorferi in culture in two independent experiments. (Sadziene, et al., J. Infect. Diseases, in press (1992). Growth inhibition titers for each of the immune sera are given in Table I below:

Table I

Mouse Strain	Vector	Titer Experiment 1	Experiment 2
BALB/C	pMV261::OspA	<8	N/A
BALB/C	pMV251::OspA	4096	8924
BALB/C	p19PS::OspA	1024	16384
BALB/C	pAB261::OspA	N/A	N/A
BALB/C	none(Control)	<8	<8
C3H/He	pMV261::OspA	32	N/A
C3H/He	pMV251::OspA	1024	32768
C3H/He	p19PS::OspA	2048	16384
C3H/He	pAB261::OspA	256	N/A
C3H/He	none(Control)	<8	<8

The above results show that antisera obtained from mice immunized with BCG transformed with pMV251::OspA or p19PS::OspA exhibited strong growth inhibition titers while sera derived from mice immunized with BCG transformed with pMV261::OspA showed lower or undetectable growth inhibition titers.

C3H/He and BALB/C mice immunized with the BCG organisms hereinabove described were then challenged with either 10^6 B. burgdorferi strain Sh^2 organisms intraperitoneally (IP) or

10^4 organisms intradermally (ID). The B.burgdorferi organisms were administered 5 weeks after a booster immunization of 10^6 transformed BCG organisms. The mice were sacrificed 14 days after the B.burgdorferi challenge, and plasma, and bladder tissue were cultured in BSKII media. (Schwan, et al., J. Clin. Microbiol, Vol. 20, pg. 155 (1984)). Cultures were monitored through day 14 by phase contrast microscopy for the presence of spirochetes. The presence of one or more spirochetes per 20 high power fields in any one of the plasma or tissue cultures was scored as an infection. The fraction of the challenged mice exhibiting positive infections in the IP, and ID challenges are given in Table II below.

Table II

Mouse Strain	Vector	No. of Infections IP	ID
BALB/C	pMV261::OspA	5/5	N/A
BALB/C	pMV251::OspA	0/5	0/5
BALB/C	p19PS::OspA	0/5	0/5
BALB/C	pAB261::OspA	4/5	N/A
BALB/C	none(Control)	4/4	4/4
C3H/He	pMV261::OspA	3/4	N/A
C3H/He	pMV251::OspA	0/5	0/5
C3H/He	p19PS::OspA	3/5	0/5
C3H/He	pAB261::OspA	3/5	N/A
C3H/He	none(Control)	5/5	5/5

The above results show that all control mice were found to be infected, whereas the mice that were immunized with BCG transformed with pMV251::OspA or p19PS::OspA were protected from infection.

It is to be understood however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced

other than as particularly described and still be within the scope of the accompanying claims.

WHAT IS CLAIMED IS:

1. An expression vector for expressing a protein or polypeptide or peptide in a bacterium, comprising:

a first DNA sequence encoding at least a secretion signal of a lipoprotein and a second DNA sequence encoding a protein or fragment thereof, or polypeptide or peptide heterologous to the bacterium which expresses the protein or fragment thereof, or polypeptide or peptide, whereby said bacterium expresses a fusion protein of a lipoprotein or lipoprotein segment and said protein or fragment thereof, or polypeptide or peptide heterologous to the bacterium which expresses the protein or polypeptide or peptide.

2. The expression vector of Claim 1 wherein the bacterium is a mycobacterium.

3. The expression vector of Claim 1 wherein said first DNA sequence encodes at least a secretion signal of a mycobacterial lipoprotein.

4. The expression vector of Claim 3 wherein said mycobacterial lipoprotein is an M. tuberculosis lipoprotein.

5. The expression vector of Claim 4 wherein said M. tuberculosis lipoprotein is selected from the group consisting of the 19 kda and 38 kda antigens.

6. The expression vector of Claim 2 wherein said vector further comprises a mycobacterial origin of replication.

7. The expression vector of Claim 2 wherein said vector further comprises a DNA sequence encoding mycobacteriophage integration into a mycobacterium chromosome.

8. The vector of Claim 1 wherein said protein or fragment thereof, or polypeptide or peptide heterologous to the bacterium which expresses the protein or fragment thereof, or polypeptide or peptide is the PspA antigen of Streptococcus pneumoniae or a fragment or derivative thereof.

9. A mycobacterium transformed with the vector of Claim 2.

10. The transformed mycobacterium of Claim 9 wherein the mycobacterium is BCG.

11. A pharmaceutical composition comprising:

the mycobacterium of Claim 9; and

an acceptable pharmaceutical carrier.

12. The expression vector of Claim 1 wherein said vector is a plasmid.

13. The vector of Claim 12 wherein the vector is a shuttle plasmid, and further comprises a bacterial origin of replication.

14. A method of protecting an animal against Lyme disease, comprising:

administering to an animal mycobacteria transformed with DNA which includes at least one DNA sequence which encodes a protein or polypeptide which elicits antibodies against Borrelia burgdorferi, said mycobacteria being administered in an amount effective to protect an animal against Lyme disease.

15. The method of Claim 14 wherein said at least one DNA sequence encodes a surface protein of Borrelia burgdorferi or a fragment or derivative thereof.

16. The method of Claim 15 wherein said surface protein of Borrelia burgdorferi is selected from the group consisting of Outer Surface Protein A and Outer Surface Protein B.

17. The method of Claim 14 wherein said mycobacteria are of the species M. bovis-BCG.

18. A composition for protecting an animal against Lyme disease, comprising:

mycobacteria transformed with DNA which includes at least one DNA sequence which encodes a protein or polypeptide which elicits antibodies against Borrelia burgdorferi; and

an acceptable pharmaceutical carrier, said mycobacteria being present in an amount effective to protect an animal against Lyme disease.

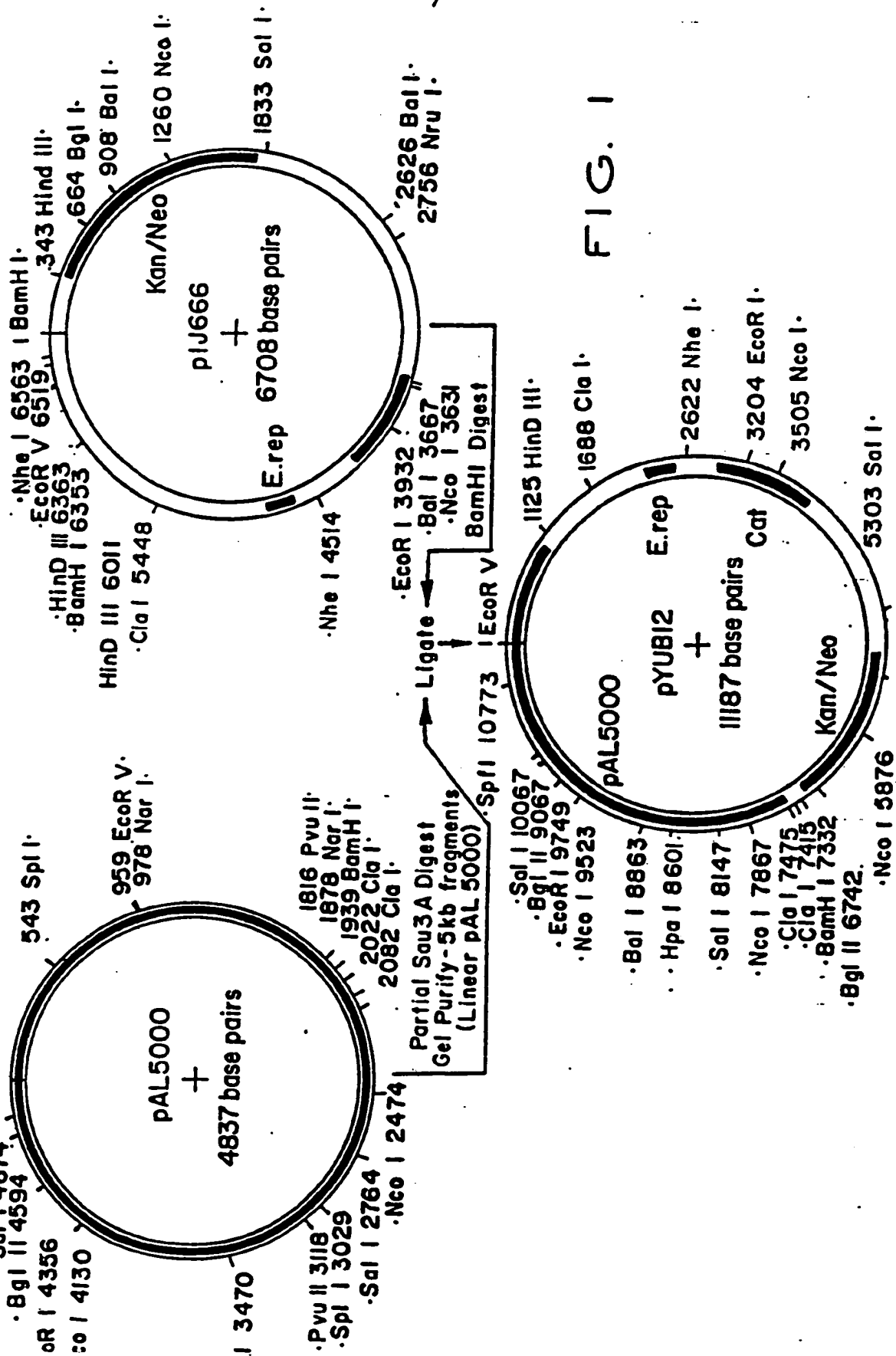
19. The composition of Claim 18 wherein said at least one DNA sequence encodes a surface protein of Borrelia burgdorferi or a fragment or derivative thereof.

20. The composition of Claim 19 wherein said surface protein of Borrelia burgdorferi is selected from the group consisting of Outer Surface Protein A and Outer Surface Protein B.

21. The composition of Claim 18 wherein said mycobacteria are of the species M. bovis-BCG.

22. An expression vector for expressing a protein or polypeptide in a bacterium which includes a DNA sequence encoding at least a secretion signal of a lipoprotein.

1/63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2626 Bal I
2756 Nru I
EcoR V 6519
EcoR I 6363
HinD III 6353
BamH I 6353
HinD III 6011
Cla I 5448
Nhe I 4514
EcoR I 3932
Bal I 3667
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+
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978 Nar I
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Gel Purify-5kb fragments
(Linear pAL 5000)
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Sal I 2764
Spl I 3029
Pvu II 3118
3470
Sal I 4674
Bgl II 4594
4356
4130
pAL5000
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Kan/Neo
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Bgl II 9067
EcoR I 9749
Nco I 9523
Bal I 8863
Hpa I 8601
Sal I 8147
Nco I 7867
Cla I 7475
Cla I 7415
BamH I 7332
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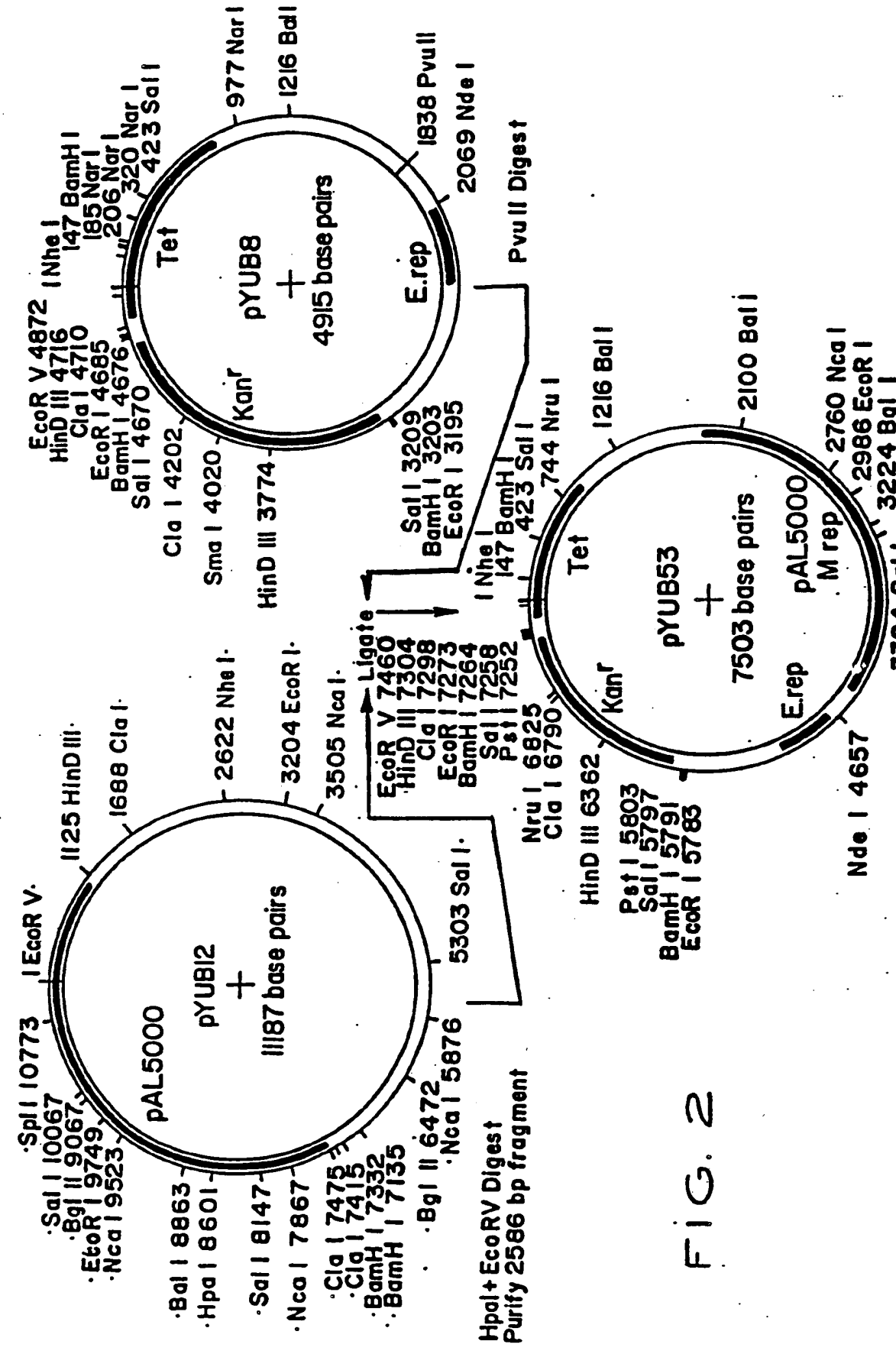
2622 Nhe I
1688 Cla I
1125 HinD III

FIG. 1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M rep
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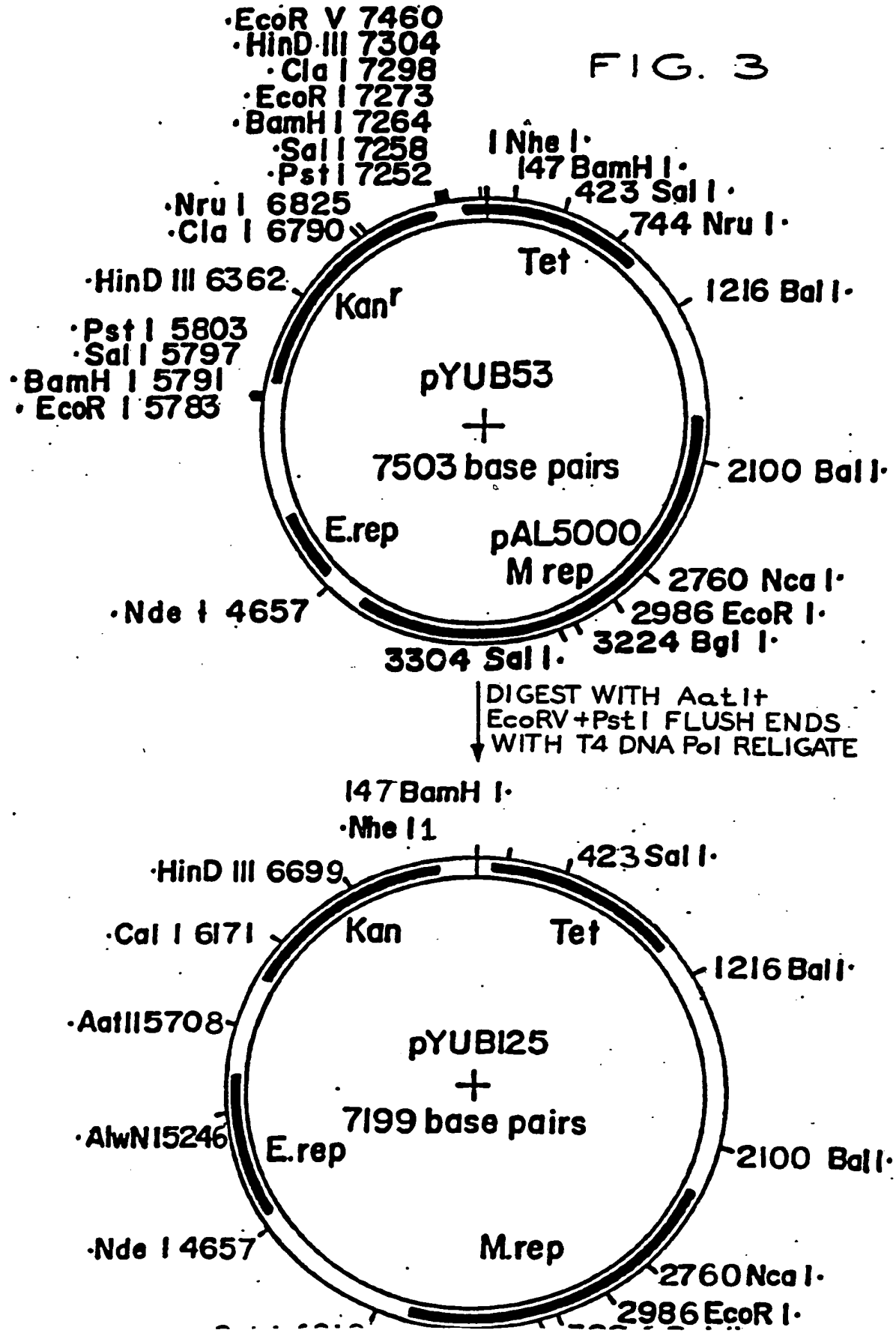
pAL5000
Rep
Ligate
Pvu II Digest
Hpa I + EcoRV Digest
Purify 2586 bp fragment

FIG. 2

FIG. 3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423 Sal I
744 Nru I
1216 Bal I
2100 Bal I
2760 Nca I
2986 EcoR I
3224 Bgl I
3304 Sal I
Nde I 4657
Nru I 6825
Cla I 6790
HinD III 6362
Pst I 5803
Sal I 5797
BamH I 5791
EcoR I 5783
Tet
Kanr
pYUB53
+
7503 base pairs
E.rep
pAL5000
M rep
DIGEST WITH AatII+
EcoRV+PstI FLUSH ENDS
WITH T4 DNA Pol RELIGATE
147 BamH I
Nhe I 1
423 Sal I
HinD III 6699
Cal I 6171
Kan
Tet
1216 Bal I
AatII 5708
pYUB125
+
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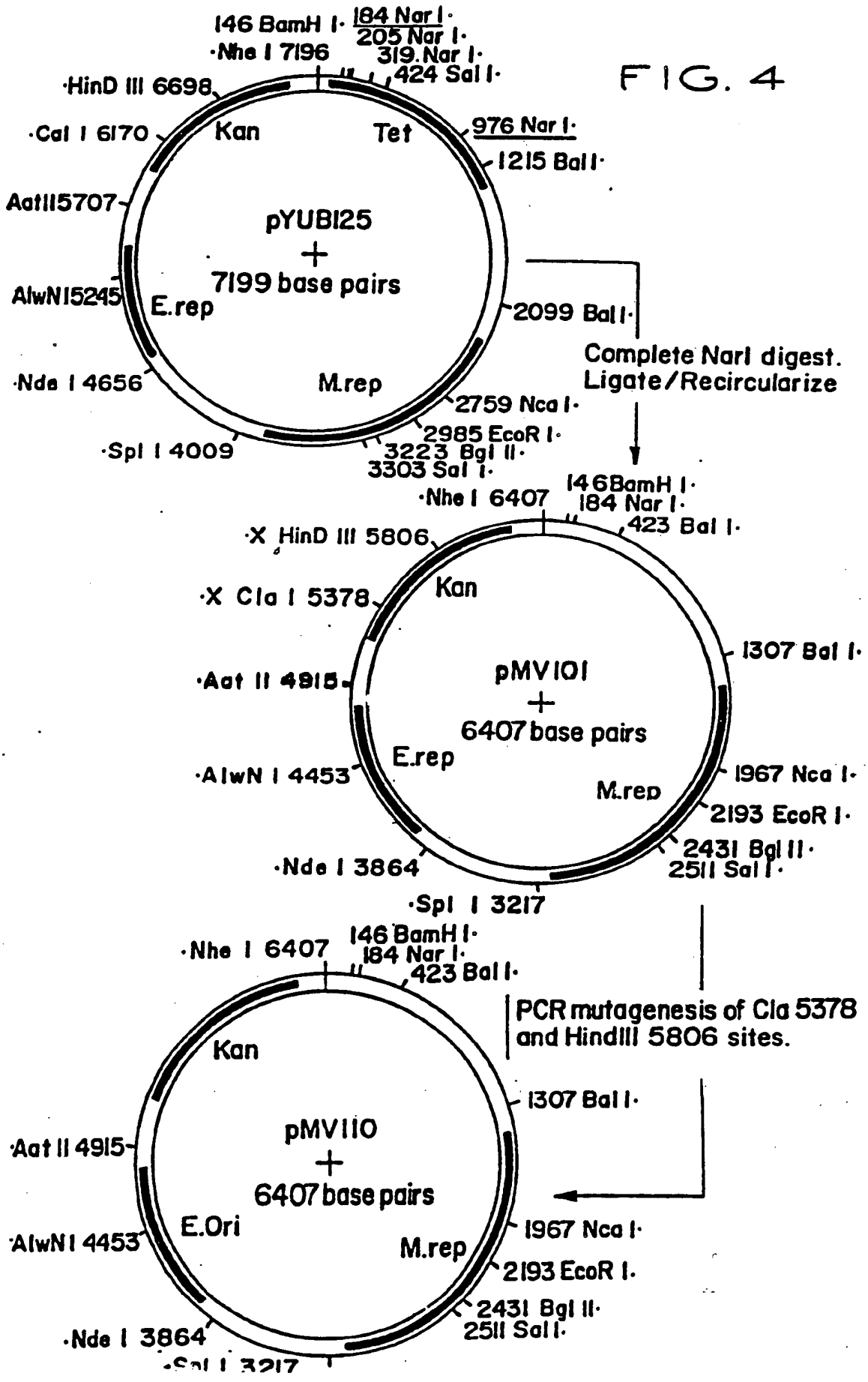
2986 EcoR I

FIG. 4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Nhe I 7196
319 Nar I
424 Sal I
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Cal I 6170
Kan
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1215 Ball
AatII5707
pYUB125
+
7199 base pairs
AlwNI5245
E.rep
2099 Ball
Complete NarI digest.
Ligate/Recircularize
Nde I 4656
M.rep
2759 Nca I
2985 EcoR I
Spl I 4009
3223 Bgl II
3303 Sal I
146BamH I
Nhe I 6407
184 Nar I
423 Bal I
X HinD III 5806
Kan
X Cla I 5378
1307 Bal I
Aat II 4915
pMV101
+
6407 base pairs
E.rep
AlwN I 4453
1967 Nca I
M.rep
2193 EcoR I
2431 Bgl II
Nde I 3864
2511 Sal I
Spl I 3217
Nhe I 6407
146 BamH I
184 Nar I
423 Bal I
PCR mutagenesis of Cla 5378
and HindIII 5806 sites.
Kan
1307 Bal I
pMV110
+
6407 base pairs
Aat II 4915
E.Ori
AlwNI 4453
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M.rep
2193 EcoR I
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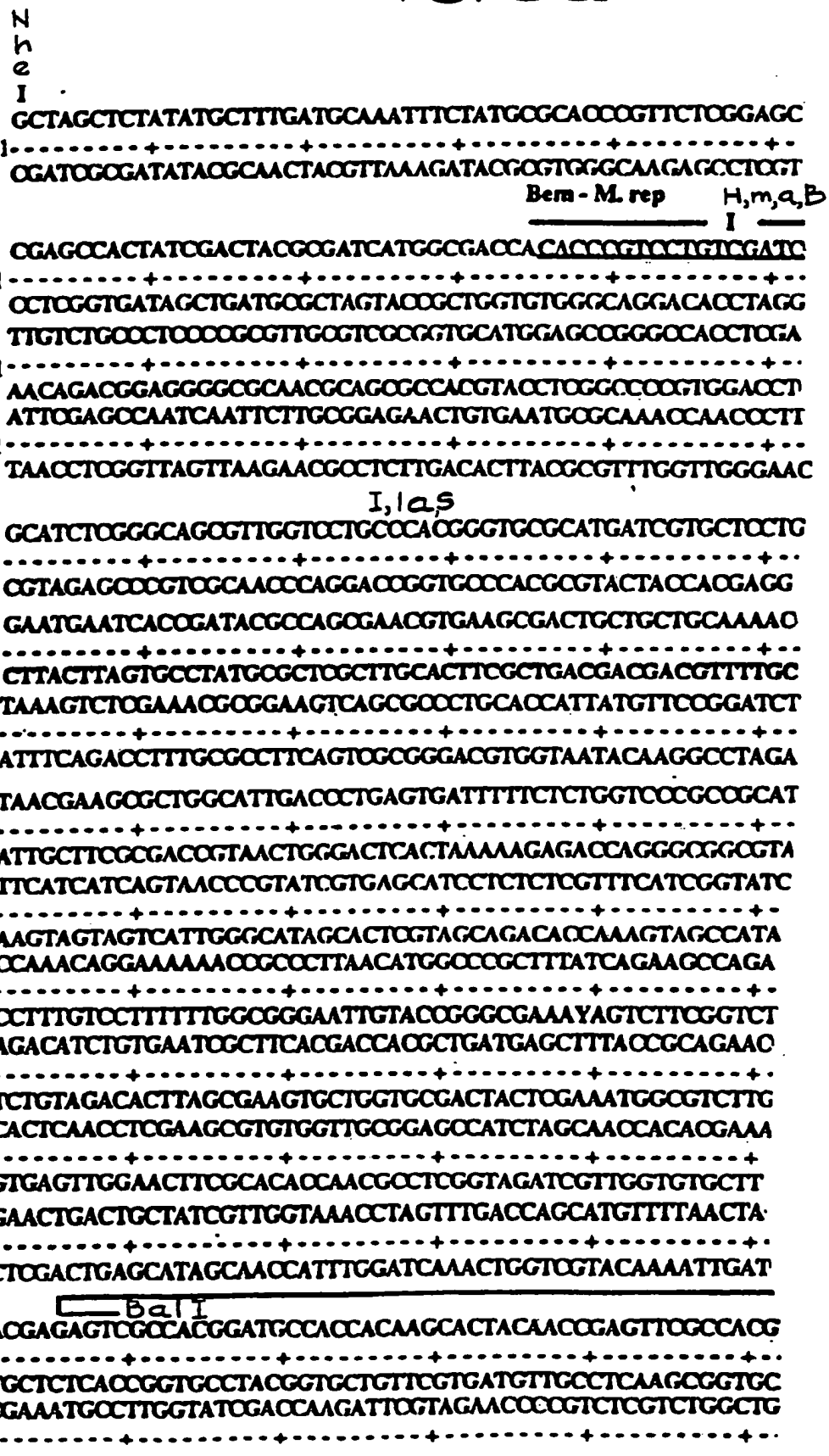
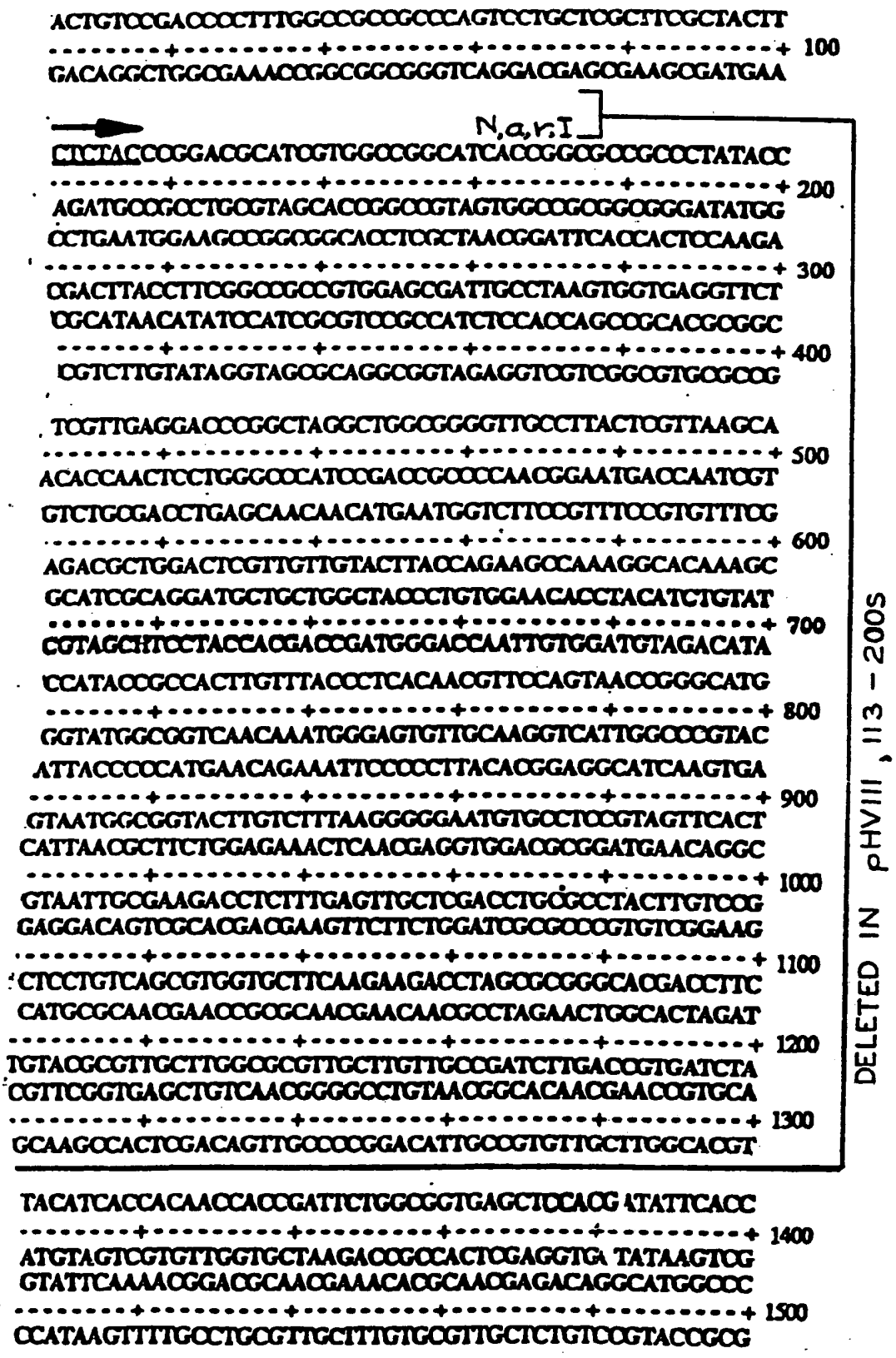
2511 Sal I
Nde I 3864
Spl I 3217

FIG. 5a



MATCH WITH FIG. 5b

FIG. 5b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CTCTACCCGGACGCATCGTGGCCGGCATCACCGGCGCCGCCCTATACC
200
AGATGCCGCCTGCGTAGCACCGGCCGTAGTGGCCGCGGCGGGATATGG
CCTGAATGGAAGCCGGCGGCACCTCGCTAACGGATTCACCACTCCAAGA
300
CGACTTACCTTCGGCCGCCGTGGAGCGATTGCCTAAGTGGTGAGGTTCT
CGCATAACATATCCATCGCGTCCGCCATCTCCACCAGCCGCACGCGGC
400
CGTCTTGTATAGGTAGCGCAGGCGGTAGAGGTCGTCGGCGTGCGCCG
TCGTTGAGGACCCGGCTAGGCTGGCGGGGTTGCCTTACTCGTTAAGCA
500
ACACCAACTCCTGGGCCCATCCGACCGCCCCAACGGAATGACCAATCGT
GTCTGCGACCTGAGCAACAACATGAATGGTCTTCCGTTTCCGTGTTTCG
600
AGACGCTGGACTCGTTGTTGTACTTACCAGAAGCCAAAGGCACAAAGC
GCATCGCAGGATGCTGCTGGCTACCCTGTGGAACACCTACATCTGTAT
700
CGTAGCCTTCCTACCACGACCGATGGGACCAATTGTGGATGTAGACATA
CCATACCGCCACTTGTTTACCCTCACAACGTTCCAGTAACCGGGCATG
800
GGTATGGCCGTCAACAAATGGGAGTGTTGCAAGGTCATTGGCCCGTAC
ATTACCCCCATGAACAGAAATTCCCCCTTACACGGAGGCATCAAGTGA
900
GTAATGGCGGTACTTGTCTTTAAGGGGGAATGTGCCTCCGTAGTTCACT
CATTAACGCTTCTGGAGAAACTCAACGAGGTGGACGCGGATGAACAGGC
1000
GTAATTGCGAAGACCTCTTTGAGTTGCTCGACCTGCGCCTACTTGTCCG
GAGGACAGTCGCACGACGAAGTTCTTCTGGATCGCGCCCGTGTCGGAAG
1100
CTCCTGTCAGCGTGGTGCTTCAAGAAGACCTAGCGCGGGCACGACCTTC
CATGCGCAACGAACCGCGCAACGAACAACGCCTAGAACTGGCACTAGAT
1200
TGTACGCGTTGCTTGGCGCGTTGCTTGTTGCCGATCTTGACCGTGATCTA
CGTTCGGTGAGCTGTCAACGGGGCCTGTAACGGCACAACGAACCGTGCA
1300
GCAAGCCACTCGACAGTTGCCCCGGACATTGCCGTGTTGCTTGGCACGT
TACATCACCACAACCACCGATTCTGGCGGTGAGCTCCACG ATATTCACC
1400
ATGTAGTCGTGTTGGTGCTAAGACCGCCACTCGAGGTGA TATAAGTCG
GTATTCAAAACGGACGCAACGAAACACGCAACGAGACAGGCATGGCCC
1500
CCATAAGTTTTGCCTGCGTTGCTTGTGCGTTGCTCTGTCCGTACCGCG
MATCH WITH FIG. 5a
DELETED IN pHV111, 113 – 200S

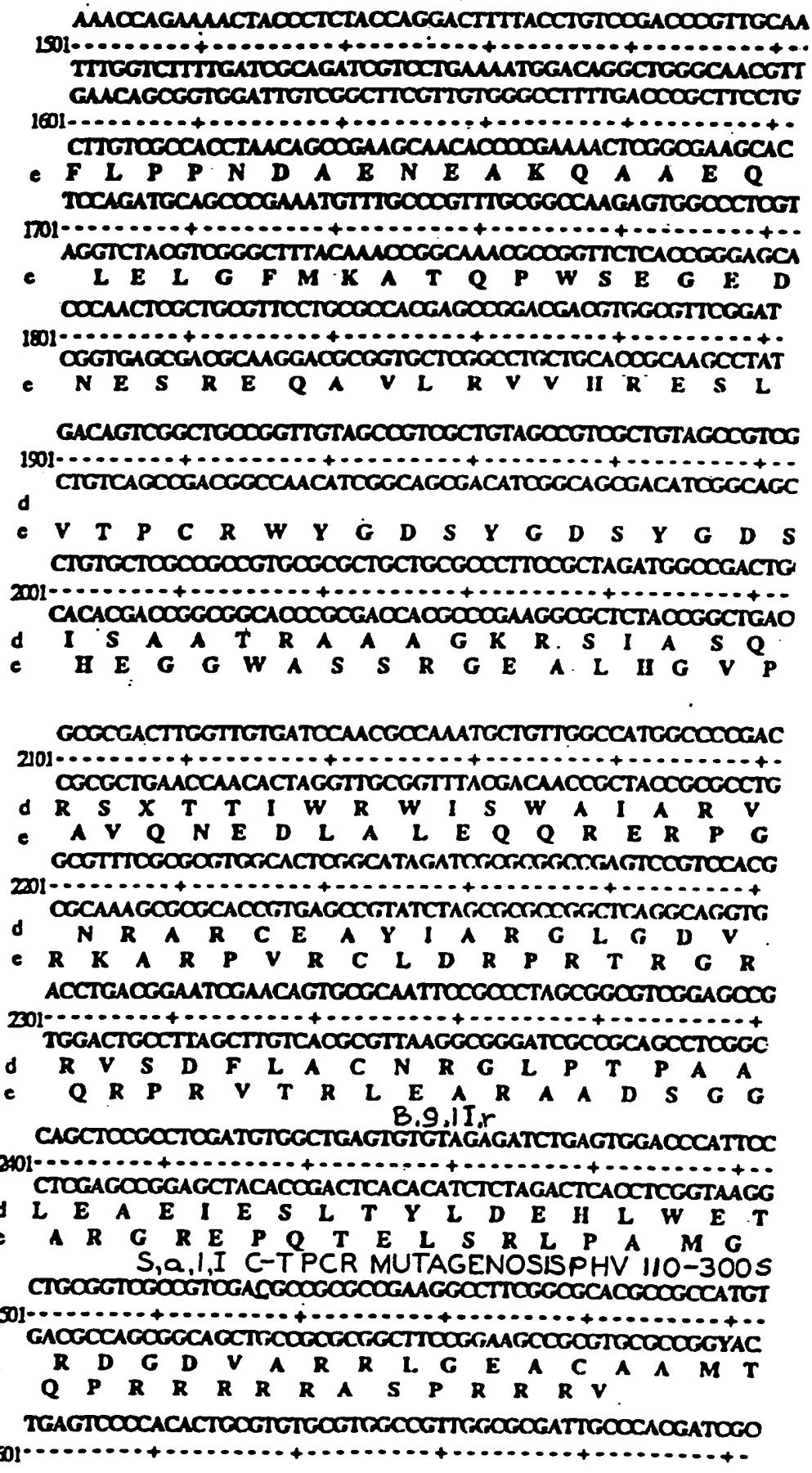
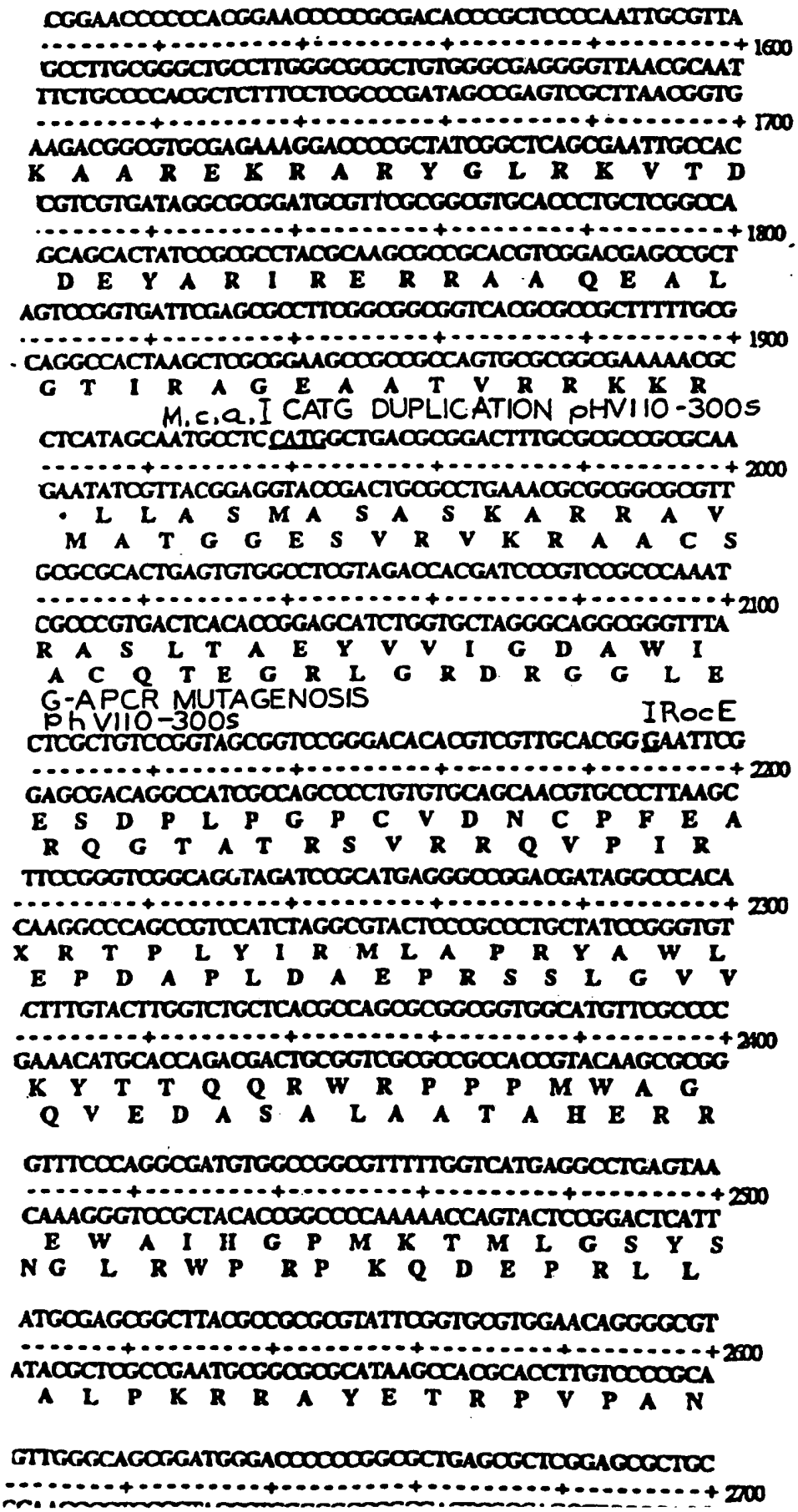


FIG. 5A a

MATCH WITH FIG. 5Ab



MATCH WITH FIG. 5Ad

FIG. 5Ab

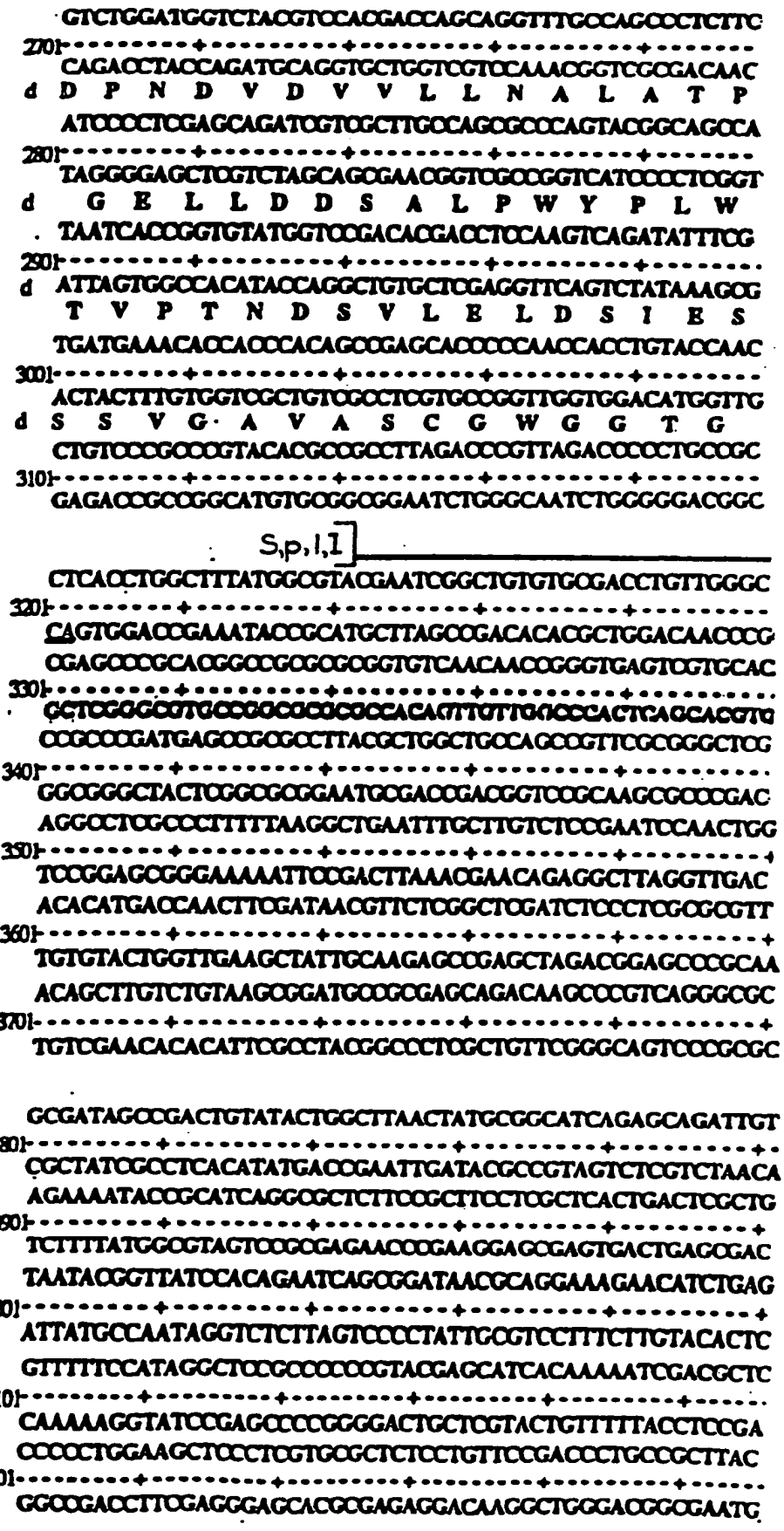


FIG. 5Bd

MATCH WITH FIG. 5Bb

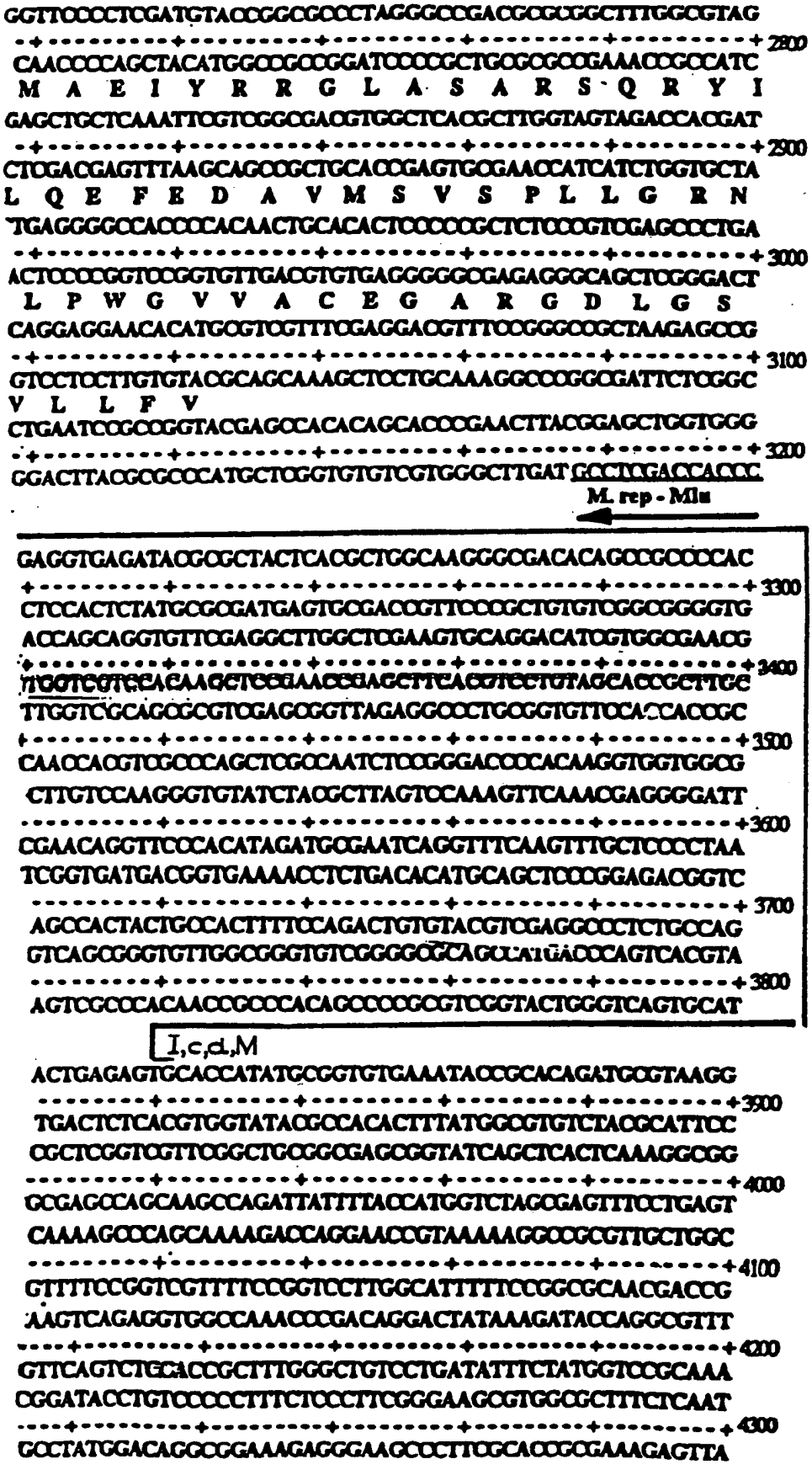
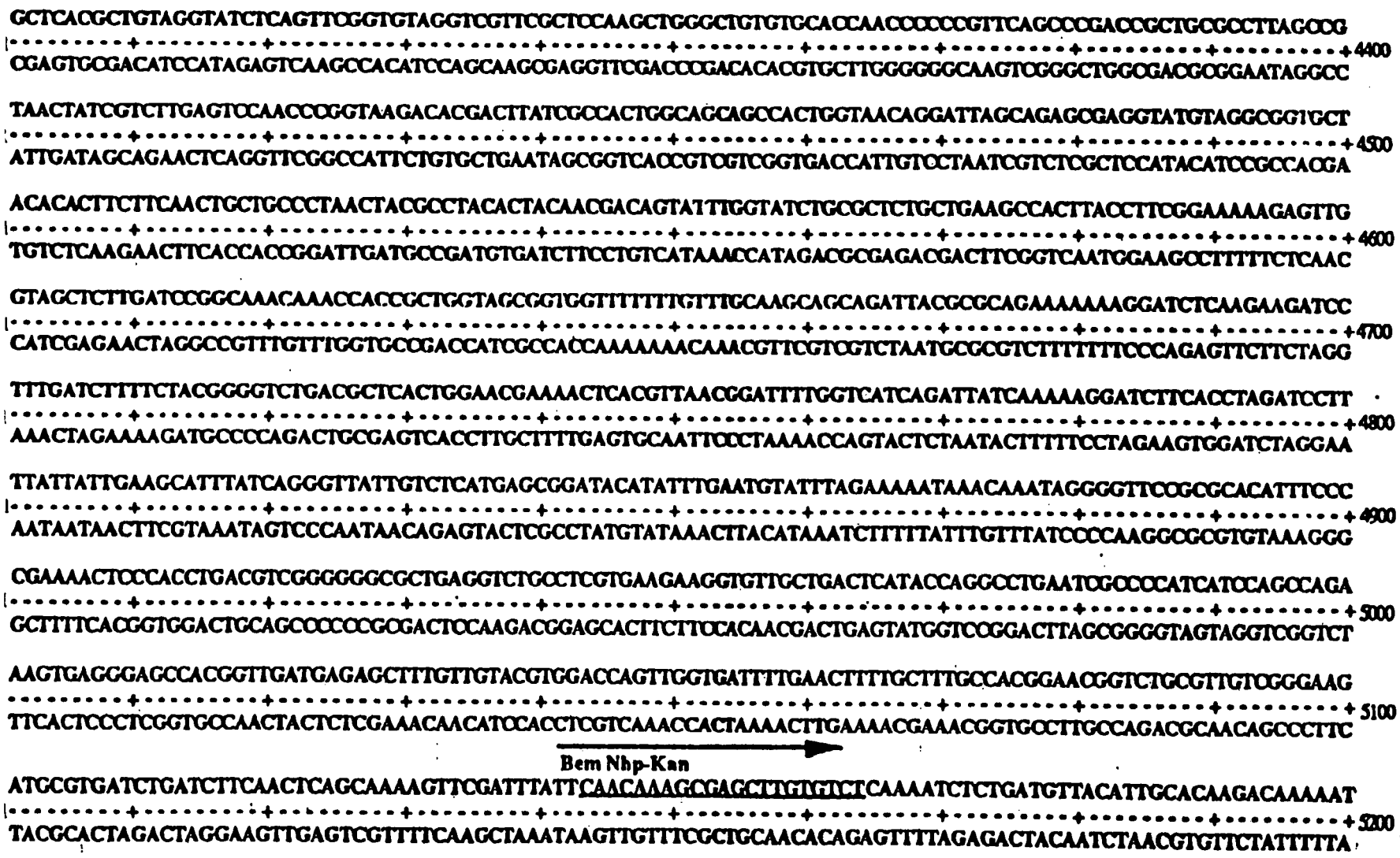


FIG. 5Bb MATCH WITH FIG. 5Ba

FIG. 5Ca



MATCH WITH FIG. 5Cb

FIG. 5Cb

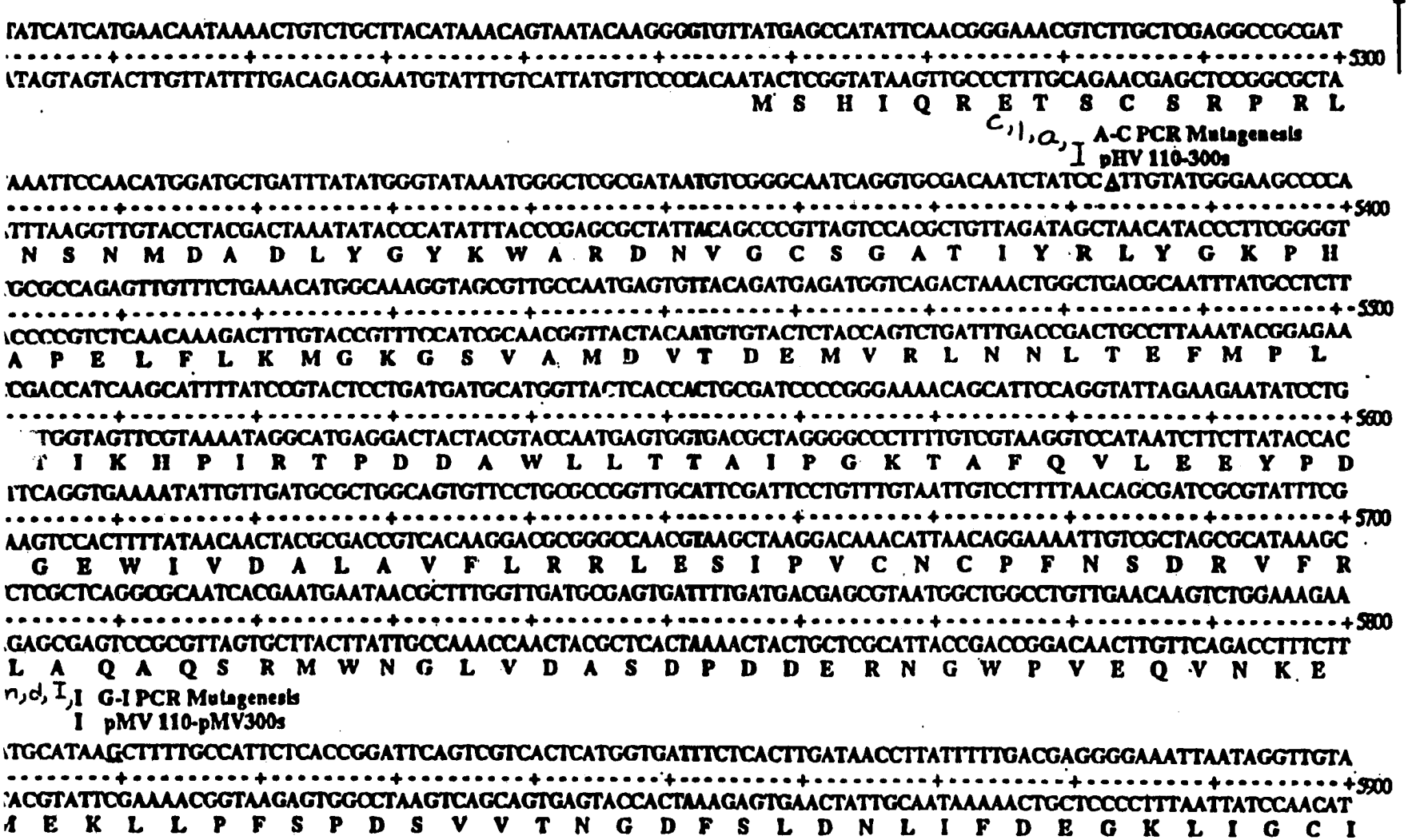

MATCH WITH FIG. 5Ca
c,l,a,I A-C PCR Mutagenesis
pHV 110-300s
n,d,I,I G-I PCR Mutagenesis
I pMV 110-pMV300s

FIG. 5D

FIG. 6

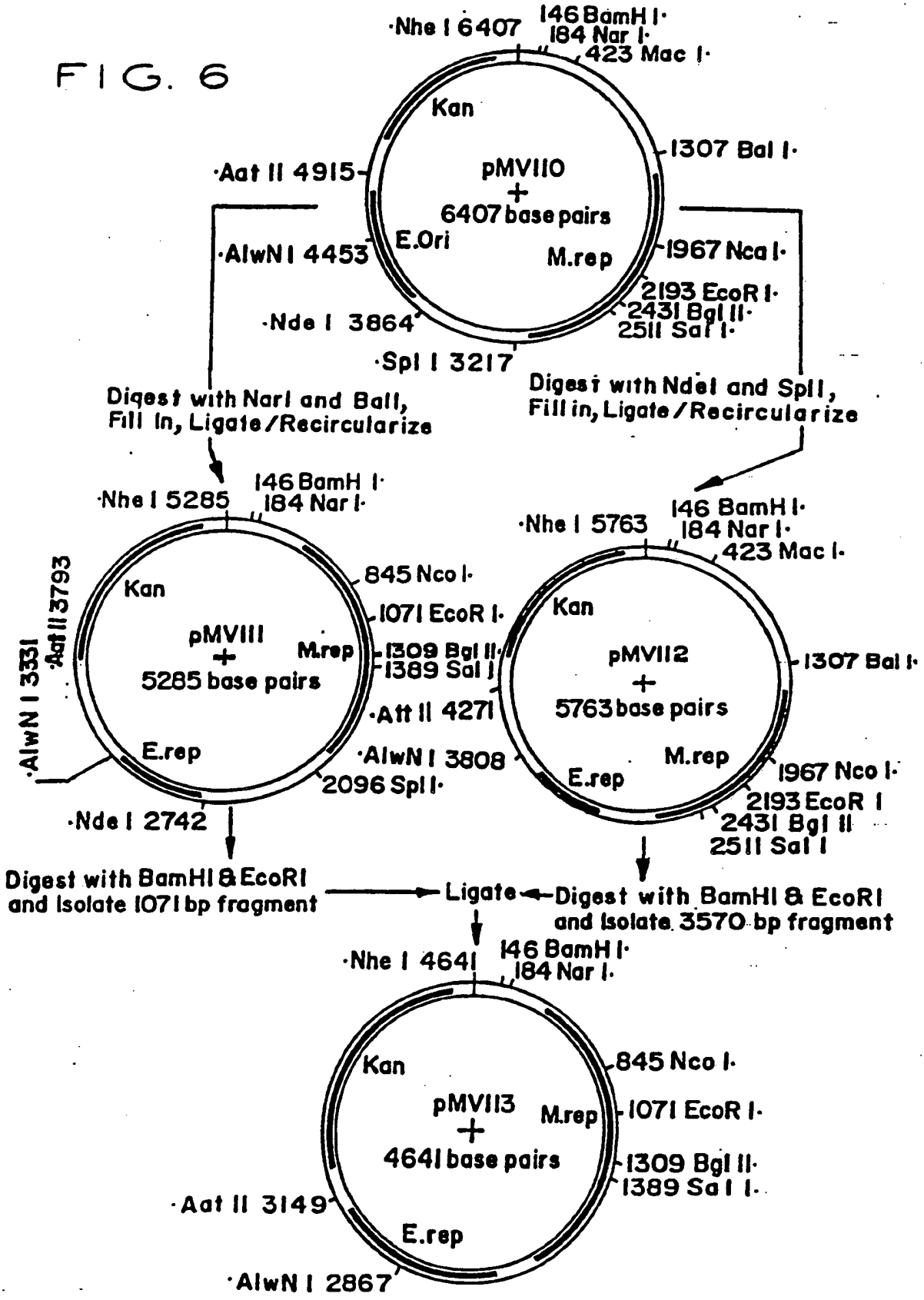
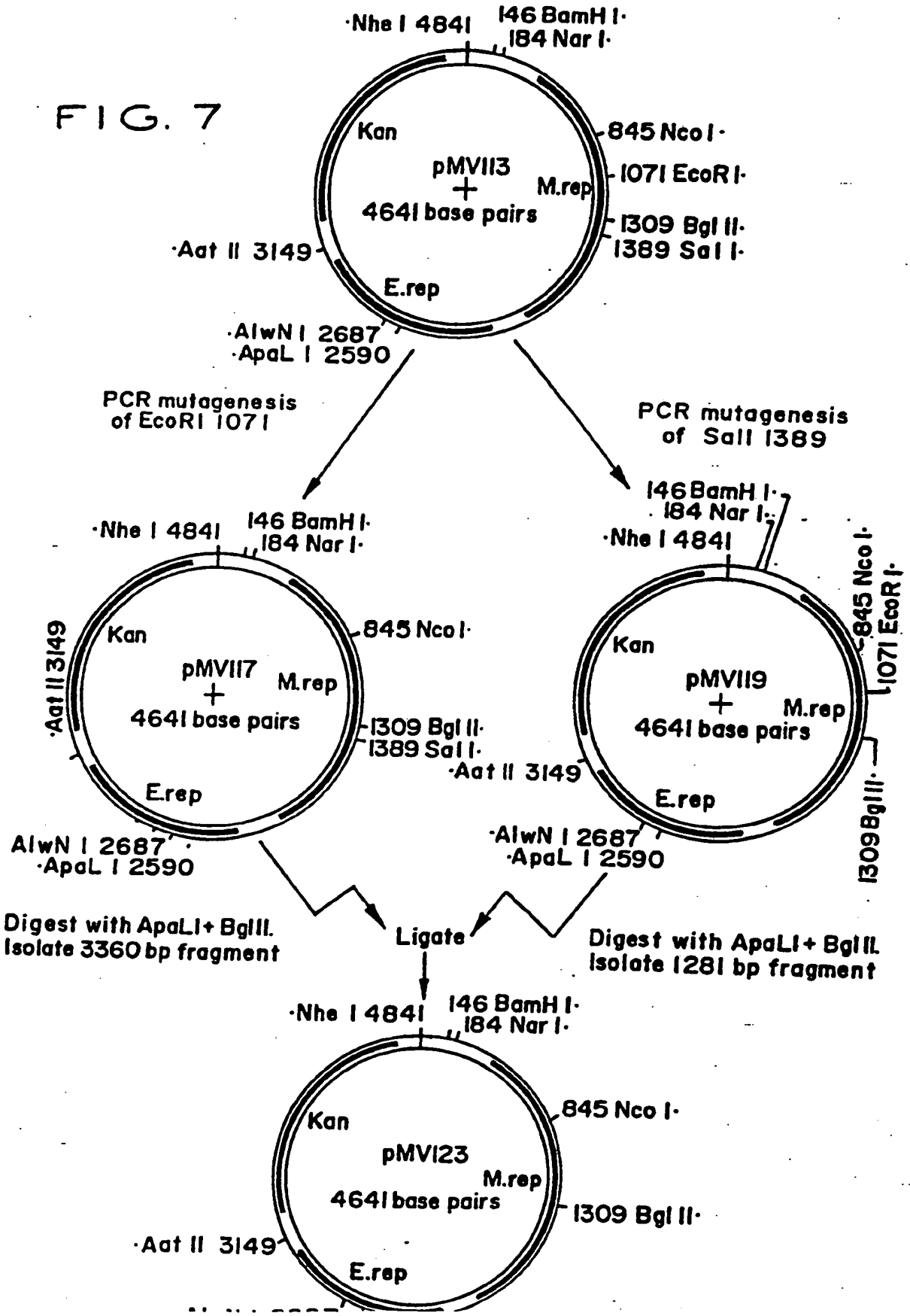

Nhe I 6407
146 BamH I
184 Nar I
423 Mac I
Kan
1307 Bal I
Aat II 4915
pMV110
+
6407 base pairs
AlwN I 4453
E.Ori
M.rep
1967 Nca I
2193 EcoR I
2431 Bgl II
2511 Sal I
Nde I 3864
Spl I 3217
Digest with NarI and BalI, Fill In, Ligate/Recircularize
Digest with NdeI and SplI, Fill in, Ligate/Recircularize
Nhe I 5285
146 BamH I
184 Nar I
Aat II 3793
845 Nco I
Kan
1071 EcoR I
pMV111
+
5285 base pairs
M.rep
1309 Bgl II
1389 Sal I
Aat II 3331
Att II 4271
AlwN I 3808
E.rep
2096 Spl I
Nde I 2742
Nhe I 5763
146 BamH I
184 Nar I
423 Mac I
Kan
pMV112
+
5763 base pairs
1307 Bal I
M.rep
E.rep
1967 Nco I
2193 EcoR I
2431 Bgl II
2511 Sal I
Digest with BamHI & EcoRI and Isolate 1071 bp fragment
Ligate
Digest with BamHI & EcoRI and Isolate 3570 bp fragment
Nhe I 4641
146 BamH I
184 Nar I
Kan
845 Nco I
pMV113
+
4641 base pairs
M.rep
1071 EcoR I
1309 Bgl II
1389 Sal I
Aat II 3149
E.rep
AlwN I 2867

FIG. 7


Nhe I 4841
146 BamH I
184 Nar I
Kan
pMV113
+
4641 base pairs
M.rep
845 Nco I
1071 EcoR I
1309 Bgl II
1389 Sal I
Aat II 3149
E.rep
AlwN I 2687
ApaL I 2590
PCR mutagenesis of EcoRI 1071
PCR mutagenesis of SalI 1389
Nhe I 4841
146 BamH I
184 Nar I
Kan
pMV117
+
4641 base pairs
M.rep
845 Nco I
1309 Bgl II
1389 Sal I
Aat II 3149
E.rep
AlwN I 2687
ApaL I 2590
Nhe I 4841
146 BamH I
184 Nar I
Kan
pMV119
+
4641 base pairs
M.rep
845 Nco I
1071 EcoR I
Aat II 3149
E.rep
AlwN I 2687
ApaL I 2590
1309 BglII
Digest with ApaLI + BglII. Isolate 3360 bp fragment
Ligate
Digest with ApaLI + BglII. Isolate 1281 bp fragment
Nhe I 4841
146 BamH I
184 Nar I
Kan
pMV123
M.rep
4641 base pairs
845 Nco I
1309 Bgl II
Aat II 3149
E.rep

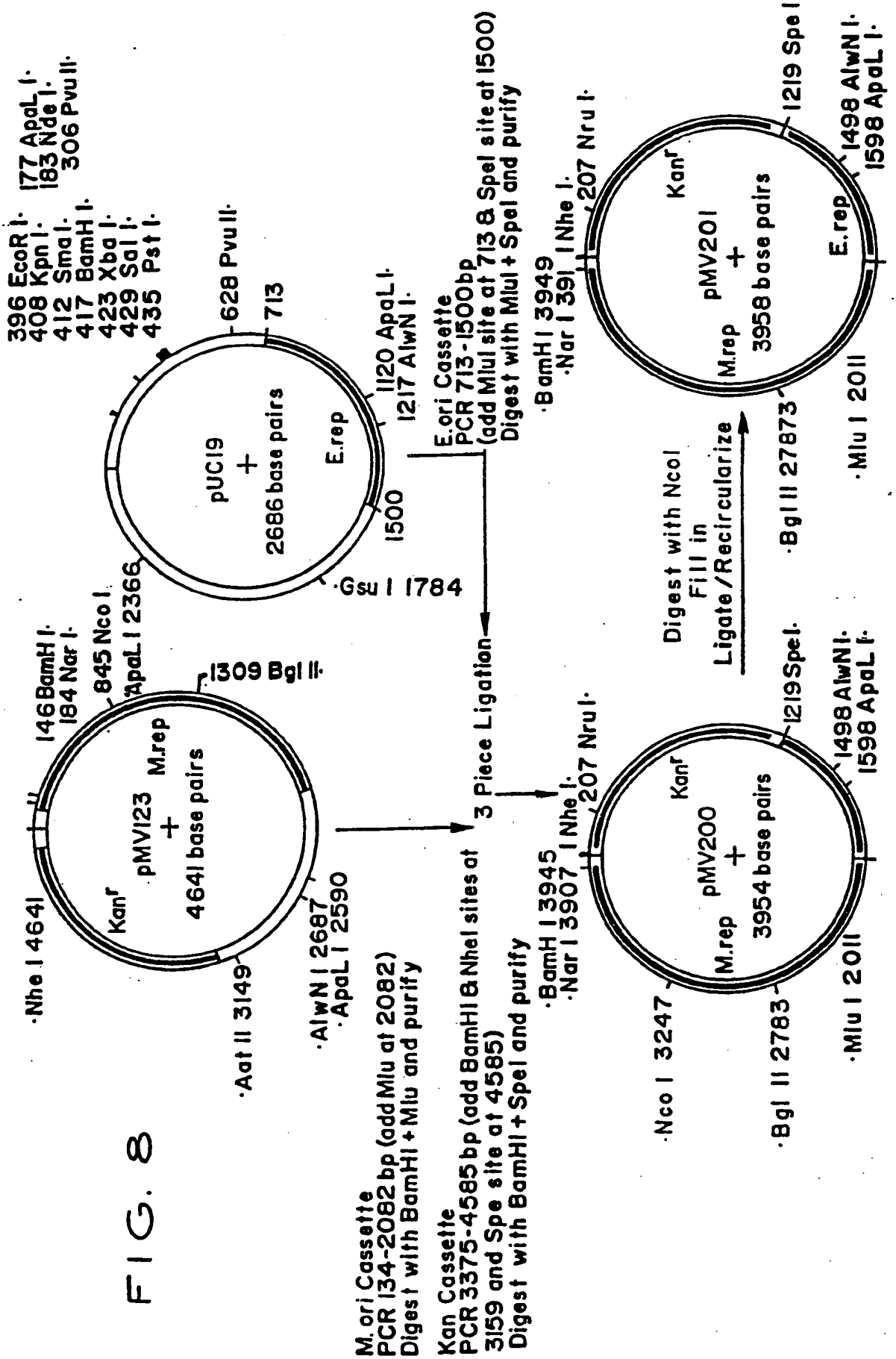

pUC19
+
2686 base pairs
E.rep
396 EcoR I
408 Kpn I
412 Sma I
417 BamH I
423 Xba I
429 Sal I
435 Pst I
628 Pvu II
713
1120 ApaL I
1217 AlwN I
1500
Gsu I 1784
E.ori Cassette
PCR 713-1500bp
(add MluI site at 713 & SpeI site at 1500)
Digest with MluI + SpeI and purify
pMV123
+
4641 base pairs
M.rep
Kan^r
Nhe I 4641
146 BamH I
184 Nar I
845 Nco I
ApaL I 2366
1309 Bgl II
Aat II 3149
AlwN I 2687
ApaL I 2590
M.ori Cassette
PCR 134-2082 bp (add Mlu at 2082)
Digest with BamHI + Mlu and purify
3 Piece Ligation
Kan Cassette
PCR 3375-4585 bp (add BamHI & NheI sites at
3159 and Spe site at 4585)
Digest with BamHI + SpeI and purify
BamH I 3945
Nar I 3907
Nhe I
207 Nru I
pMV200
+
3954 base pairs
M.rep
Kan^r
Nco I 3247
Bgl II 2783
1219 SpeI
1498 AlwN I
1598 ApaL I
Mlu I 2011
Digest with NcoI
Fill in
Ligate/Recircularize
BamH I 3949
Nar I 391
Nhe I
207 Nru I
pMV201
+
3958 base pairs
M.rep
Kan^r
Bgl II 27873
1219 Spe I
1498 AlwN I
1598 ApaL I
Mlu I 2011
E.rep

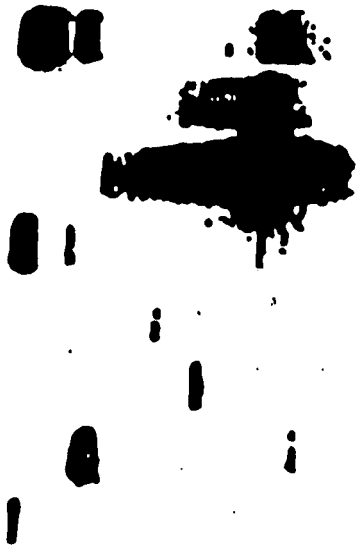
FIG. 8

FIG. 9

SYNTHETIC MULTIPLE CLONING SITE (MCS) + STRAND

GAA GGC GCG GCC GCG GTA CCA GAT CTT TAA ATC TAG ATA TCC ATG GAT
CCA GCT GCA GAA TTC GAA GCT TAT CGA TGT CGA CGT AGT TAA CTA GCG
TAC GAT CGA CTG CCA GGC ATC AAA TAA AAC GAA AGG CTC AGT CGA AAG
AGT GGG CCT TTC GTT TTA TCT GTT GTT TGT CCG GCC ATC ATG GCC GCG
GTG ATC AGC TAG TAC G

FIG. 44



BCG PASTEUR

M. smegmatis mc^2155

M. smegmatis mc^26

M. bovis

M. chelonei

M. leprae

M. phlei

M. tuberculosis

17/63

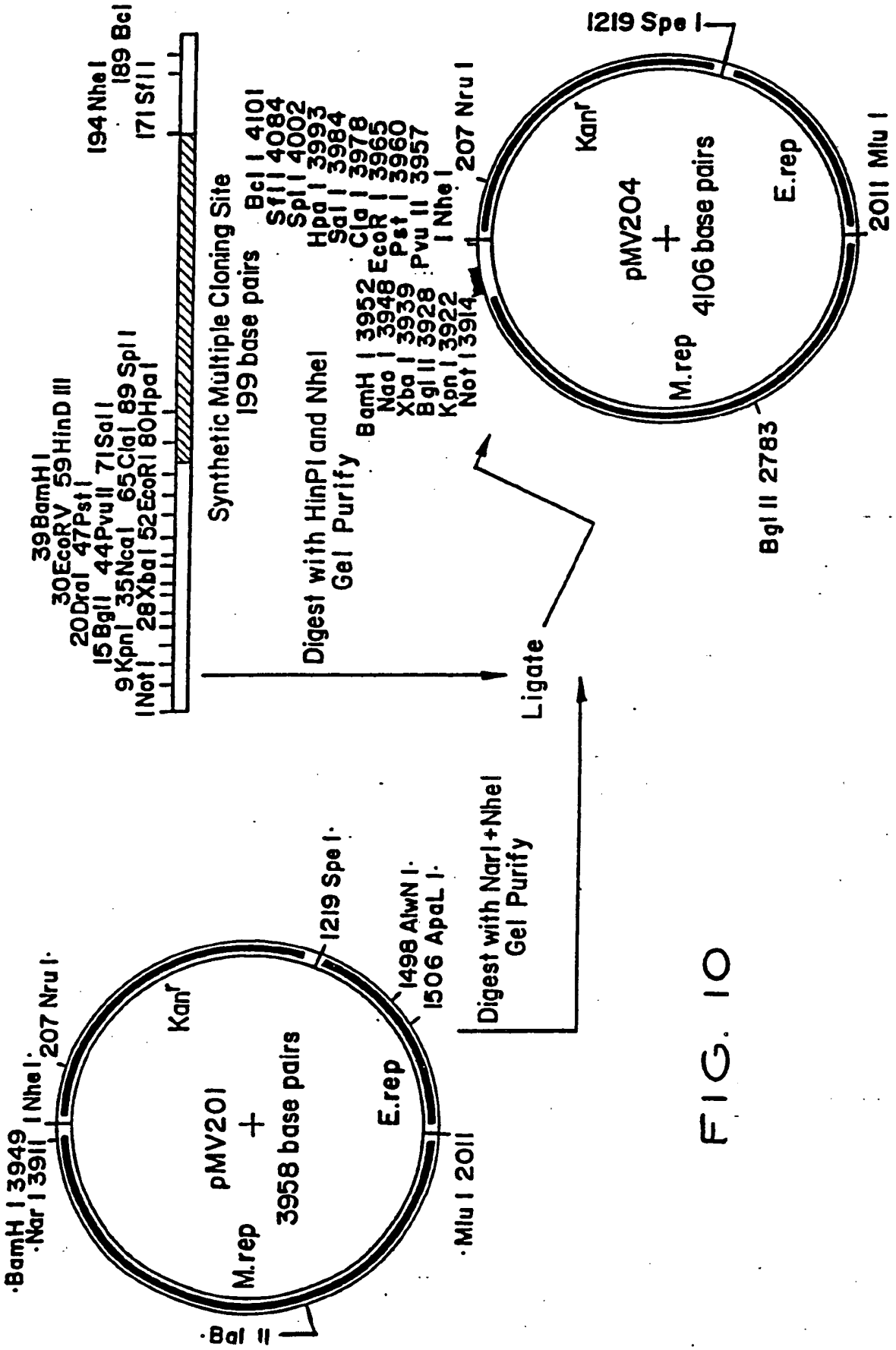

pMV201
+
3958 base pairs
M.rep
E.rep
Kan^r
BamH I 3949
Nar I 3911
Nhe I
207 Nru I
1219 Spe I
1498 AlwN I
1506 ApaL I
Mlu I 2011
Bal II
Digest with NarI+NheI
Gel Purify
Ligate
Synthetic Multiple Cloning Site
199 base pairs
194 Nhe I
189 Bcl I
171 Sfi I
39 BamH I
30 EcoRV
59 HinD III
20 Dra I
47 Pst I
15 Bgl II
44 Pvu II
71 Sal I
35 Nca I
65 Cla I
89 Spl I
9 Kpn I
28 Xba I
52 EcoR I
80 Hpa I
1 Not I
Digest with HinPI and NheI
Gel Purify
BamH I 3952
Nae I 3948
Xba I 3939
Bgl II 3928
Kpn I 3922
Not I 3914
Bcl I 4101
Sfi I 4084
Spl I 4002
Hpa I 3993
Sal I 3984
Cla I 3978
EcoR I 3965
Pst I 3960
Pvu II 3957
I Nhe I
207 Nru I
1219 Spe I
pMV204
+
4106 base pairs
M.rep
E.rep
Kan^r
Bgl II 2783
2011 Mlu I

FIG. 10

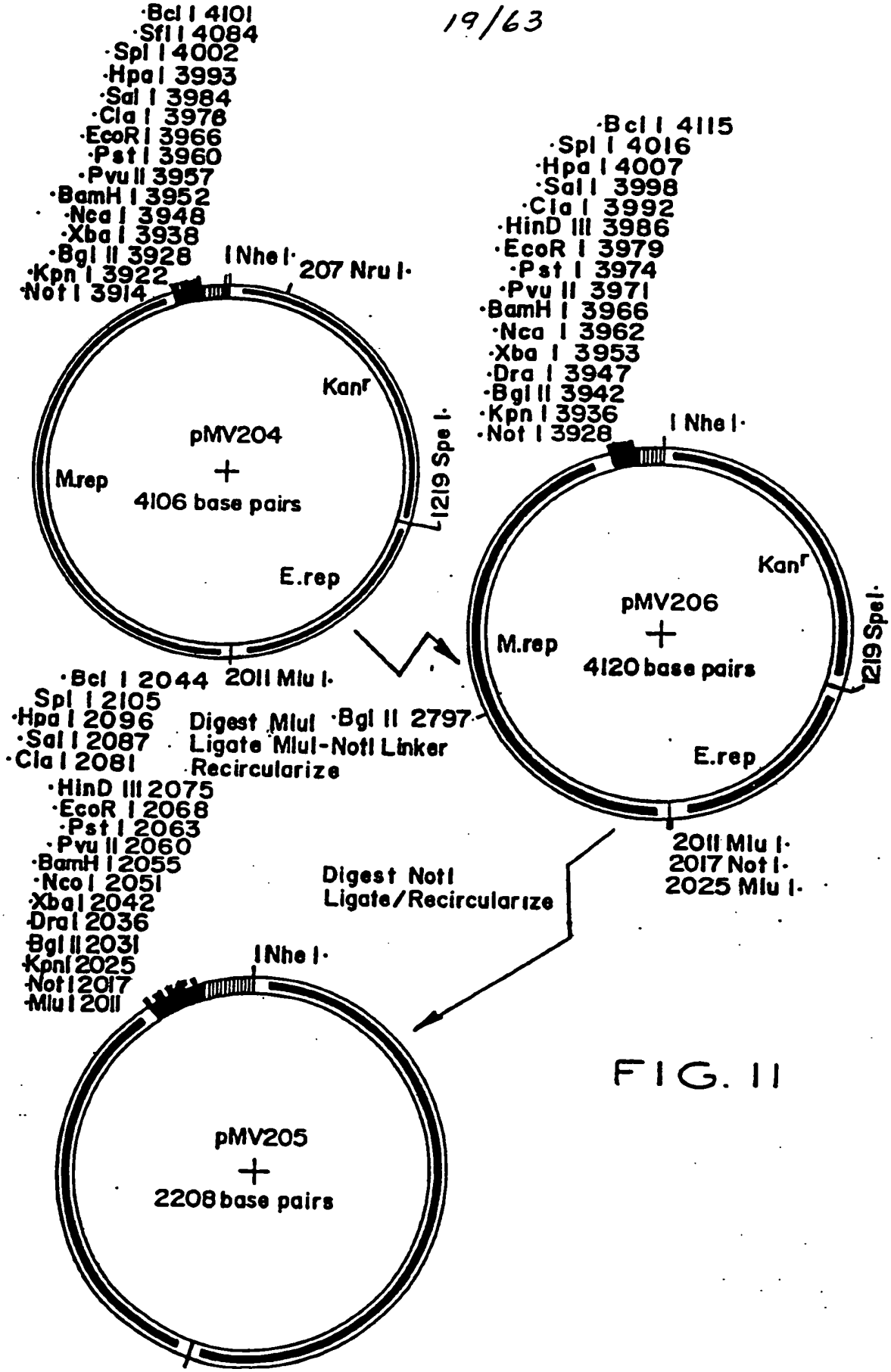

·Bcl I 4101
·Sfi I 4084
·Spl I 4002
·Hpa I 3993
·Sal I 3984
·Cla I 3978
·EcoR I 3966
·Pst I 3960
·Pvu II 3957
·BamH I 3952
·Nca I 3948
·Xba I 3938
·Bgl II 3928
·Kpn I 3922
·Not I 3914
I Nhe I·
207 Nru I·
Kanr
pMV204
+
4106 base pairs
M.rep
E.rep
1219 Spe I·
·Bcl I 4115
·Spl I 4016
·Hpa I 4007
·Sal I 3998
·Cla I 3992
·HinD III 3986
·EcoR I 3979
·Pst I 3974
·Pvu II 3971
·BamH I 3966
·Nca I 3962
·Xba I 3953
·Dra I 3947
·Bgl II 3942
·Kpn I 3936
·Not I 3928
I Nhe I·
Kanr
pMV206
+
4120 base pairs
M.rep
E.rep
1219 Spe I·
2011 Mlu I·
2017 Not I·
2025 Mlu I·
·Bcl I 2044
Spl I 2105
·Hpa I 2096
·Sal I 2087
·Cla I 2081
·HinD III 2075
·EcoR I 2068
·Pst I 2063
·Pvu II 2060
·BamH I 2055
·Nco I 2051
·Xba I 2042
·Dra I 2036
·Bgl II 2031
·Kpn I 2025
·Not I 2017
·Mlu I 2011
2011 Mlu I·
Digest MluI
Ligate MluI-NotI Linker
Recircularize
·Bgl II 2797
Digest NotI
Ligate/Recircularize
I Nhe I·
pMV205
+
2208 base pairs

FIG. 11

FIG. 12a

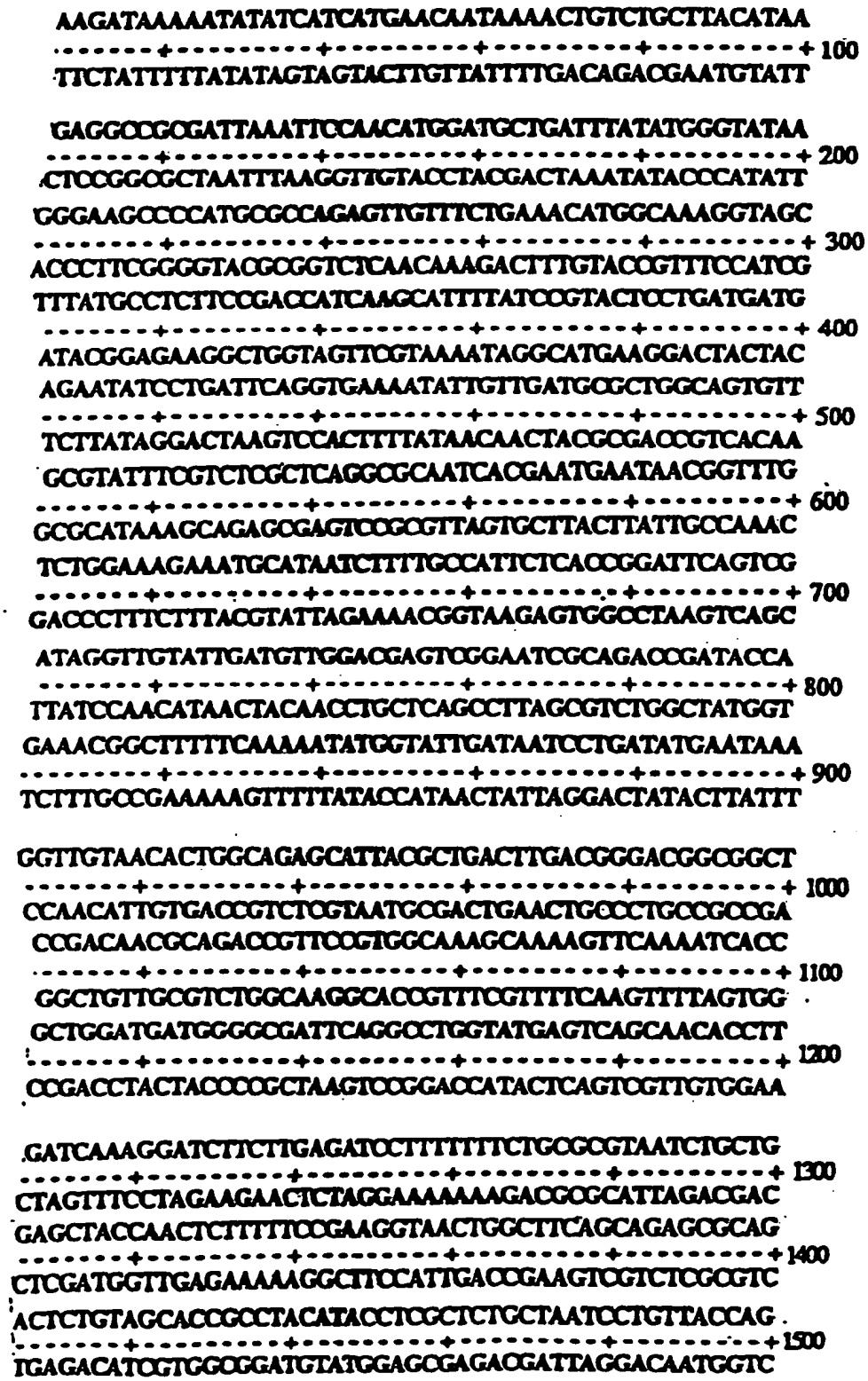
pMV 206

N
h Begin Kan Cassette
e
I

GCTAGCCAACAAAGCCGACGTTGTGTCTCAAAATCTCTGATGTTACATTGCAC
1----------+----------+----------+----------+----------+---
CGATCGGTTGTTCGCTGCAACACAGAGTTTTAGAGACTACAATGTAACCGTG
KAN START CONDON
ACAGTAATACAAGGGGTGTTATGAGCCATATTCAACGGGAAACGTCTTGCTC
101----------+----------+----------+----------+----------+---
TGTCATTATGTTCCCCACAATACTCGGTATAAGTTGCCCTTTGCAGAACGAG
NheI
ATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGTTGTAT
201----------+----------+----------+----------+----------+---
TACCCGAGCGCTATTACAGCCCGTTAGTCCACGCTGTTAGATAGCGAACAT
GTTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACTGGCTGACGGAA
301----------+----------+----------+----------+----------+---
CAACGGTTACTACAATGTCTACTCTACCAGTCTGATTTGACCGACTGCCTTAA
CATGGTTACTCACCACTGCGATCCCCGGGAAAACAGCATTCCAGGTATTAGA
401----------+----------+----------+----------+----------+---
GTACCAATGAGTGGTGACGCTAGGGGCCCTTTGTCGTAAGGTCCATAATCT
CCTGCGCCGGTTGCATTCGATTCCTGTTTGTAATTGTCCTTTTAACAGCGATC
501----------+----------+----------+----------+----------+---
GGACGCGGCCAACGTAAGCTAAGGACAAACATTAACAGGAAAATTGTCGCTA
GTTGATGCGAGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAG
601----------+----------+----------+----------+----------+---
CAACTACGCTCACTAAAACTACTGCTCGCATACCGACCGGACAACTTGTTCA
TCACTCATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAATTA
701----------+----------+----------+----------+----------+---
AGTGAGTACCACTAAAGAGTGAACTATTGGAATAAAAACTGCTCCCCTTTAA
GGATCTYGCCATCCTATGGAACTGCCTCGGTGAGTTTTCTCCTTCATTACA
801----------+----------+----------+----------+----------+---
CCTAGAACGGTAGGATACCTTGACGGAGCCACTCAAAAGAGGAAGTAATG
KAN STOP CONDON
TTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAATCAGAATTGGTTAATT
901----------+----------+----------+----------+----------+---
AACGTCAAAGTAAACTACGAGCTACTCAAAAGATTAGTCTTAACCAATTAA
TTGTTGAATAAATCGAACTTTTGCTGAGTTGAAGGATCAGATCACGCATCTTC
1001----------+----------+----------+----------+----------+---
AACAACTTATTTAGCTTGAAAACGACTCAACTTCCTAGTCTAGTGCGTAGAAG
AACTGGTCCACCTACAACAAAGCTCTCATCAACCGTGGCTCCCTCACTTTCTG
1101----------+----------+----------+----------+----------+---
TTGACCAGGTGGATGTTGTTTCGAGAGTAGTTGGCACCGAGGGAGTGAAAGA
END KAN CASSETTE I, e, P, S BEGIN E. RAP
CCTCACGAGGCAGACCTCACTAGTTCCACTGAGCGTCAGACCCCGTAGAAAA
1201----------+----------+----------+----------+----------+---
GAAGTGCTCCGTCTGGAGTGATCAAGGTGACTCGCAGTCTGGGGCATCTTTT
CTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAA
1301----------+----------+----------+----------+----------+---
GAAGATTTGTTTTTTTGGTGGCGATGGTCGCCACCAAACAAACGGCCTAGTT
ATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGA
1401----------+----------+----------+----------+----------+---
TATGGTTTATGACAGCGAAGATCACATCGGCATCAATCCGGTGGTGAAGTTCT

MATCH WITH FIG. 12b

FIG. 12b



MATCH WITH FIG. 12a

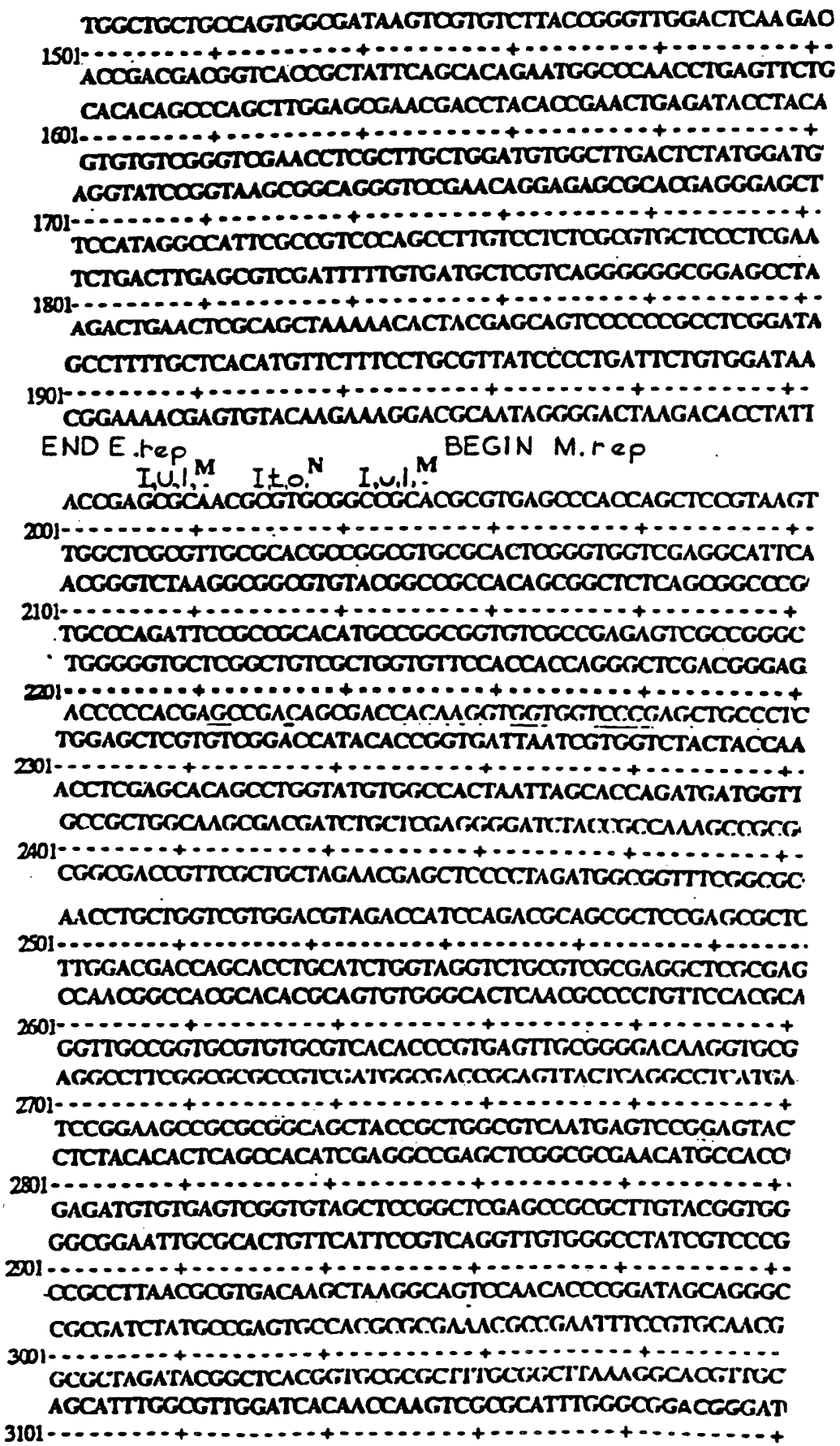
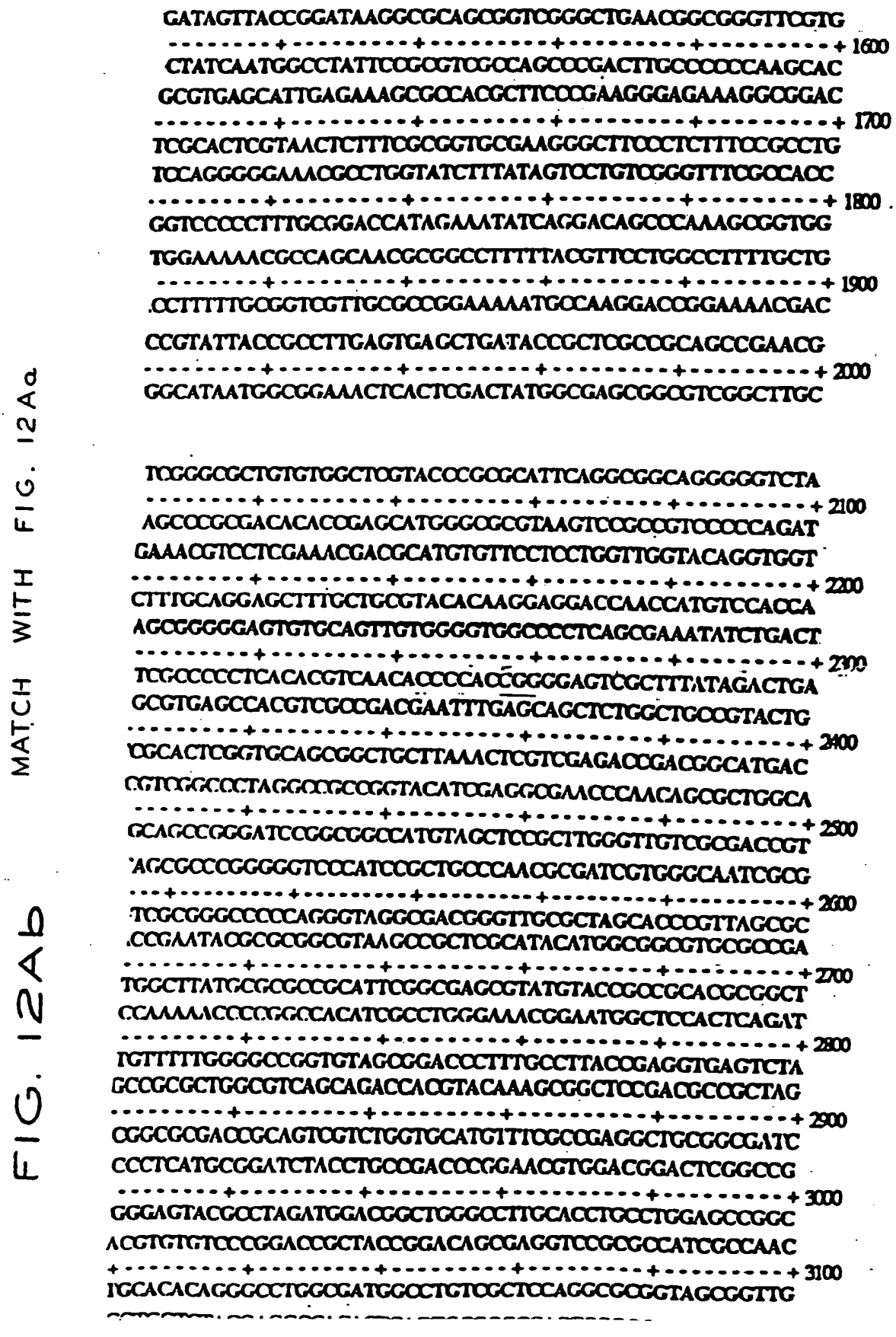


FIG. 12A MATCH WITH FIG. 12Ab



MATCH WITH FIG. 12Aa

FIG. 12Ab

```
   AGGGCGCAGCAGCGCGCACGGCGGCGAGCACAGTTGCGCGGCGCGCAAAG
3201--------+---------+---------+---------+---------+---------+--
   TCCCGCGTCGTCGCGCGTGCCGCCGCTCGTGTCAACGCGCCGCGCGTTTCA

   CGGCTACAGCGACGGCTACAACCGGCAGCCGACTGTCCGCAAAAAGCGGCG
3301--------+---------+---------+---------+---------+---------+--
   GCCGATGTCGCTGCCGATGTTGGCCGTCGGCTGACAGGCGTTTTTCGCCGC

   GTCGTCCGGCTCGTGGCGCAGGAACGCAGCGAGTGGCTCGCCGAGCAGGC
3401--------+---------+---------+---------+---------+---------+
   CAGCAGGCCGAGCACCGCGTCCTTGCGTCGCTCACCGAGCGGCTCGTCCG

   GGCCGCAAACGGCCAAACATTTCGGGCTGCATCTGGACACCGTTAAGCGA
3501--------+---------+---------+---------+---------+---------+
   CCGGCGTTTGCCGGTTTGTAAAGCCCGACGTAGACCTGTGGCAATTCGCT
   AAAGGCCCACAACGAAGCCGACAATCCACCGCTGTTCTAACGCAATTGG
3601--------+---------+---------+---------+---------+--------
   TTTCCGGGTGTTGCTTCGGCTGTTAGGTGGCGACAAGATTGCGTTAAC

   CAGGTAAAAGTCCTGGTAGACGCTAGTTTTCTGGTTTGGGCCATGCCT
3701--------+---------+---------+---------+---------+--------
   GTCCATTTTCAGGACCATCTGCGATCAAAAGACCAAACCCGGTACGGA

   GGGTTCTACGAATCTTGGTCGATACCAAGCCATTTCCGCTGAATATCG
3801--------+---------+---------+---------+---------+--------
   CCCAAGATGCTTAGAACCAGCTATGGTTCGGTAAAGGCGACTTATAGC
```

MATCH WITH FIG. 12Bb

Multiple Cloning Site

End M. rep — N o t I — S c I I — K p n I — B g l I

```
   TTGTAGTGTTGTGGTGGCATCCGTGGCGCGGCCGCGGTACCAGATCTT
3901--------+---------+---------+---------+---------+---------
   AACATCACAACACCACCGTAGGCACCGCGCCGGCGCCATGGTCTAGAA
```

Stop Condons 3 Frames — S p I I

Begin Transcription Terminator

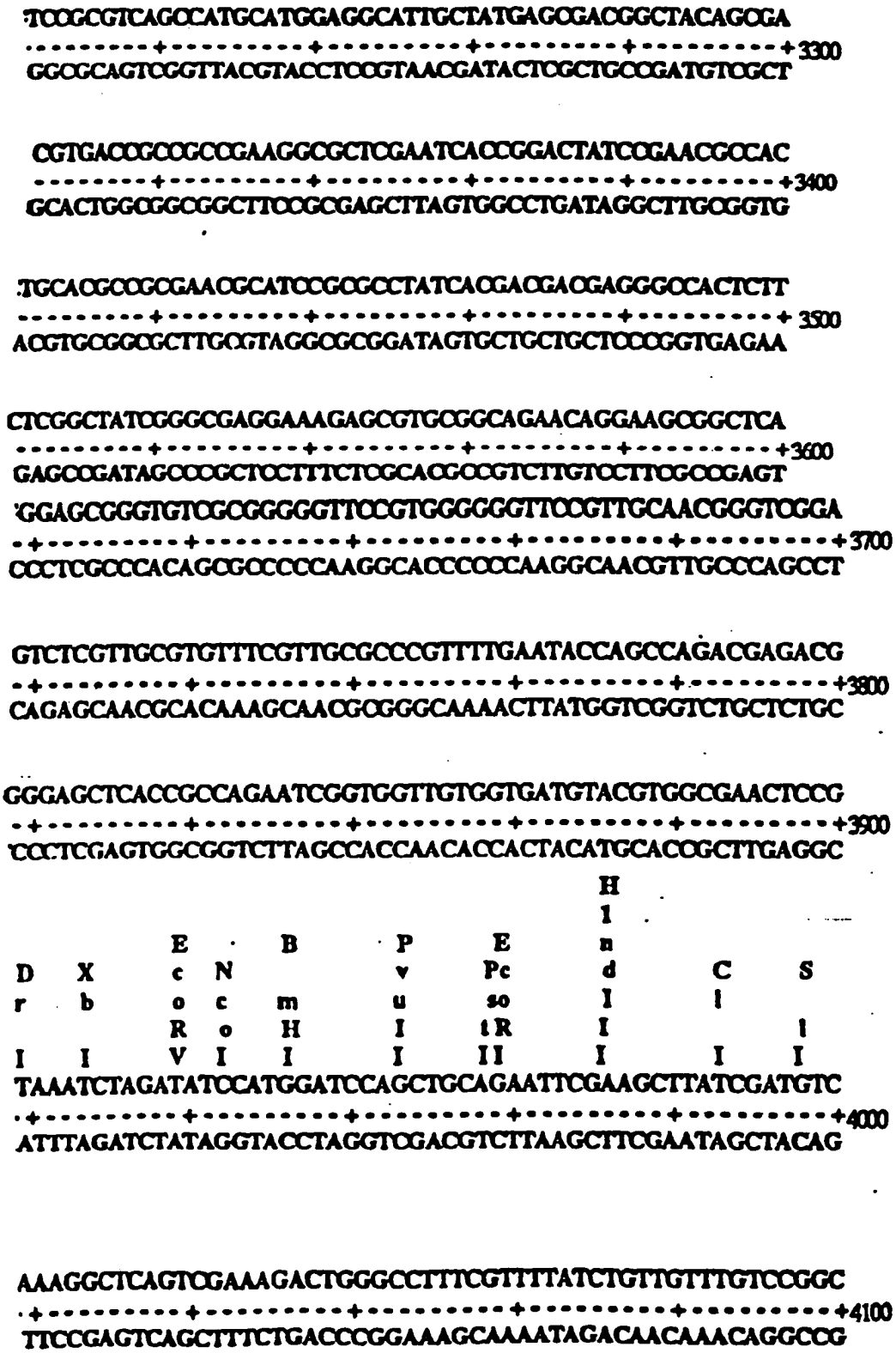
```
   GACGTAGTTAACTAGCGTACGATCGATCGCCAGGCATCAAATAAAACG
4001--------+---------+---------+---------+---------+---------
   CTGCATCAATTGATCGCATGCTAGCTGACGGTCCGTAGTTTATTTTGCT
```

S f l I — S a c I — B c l I

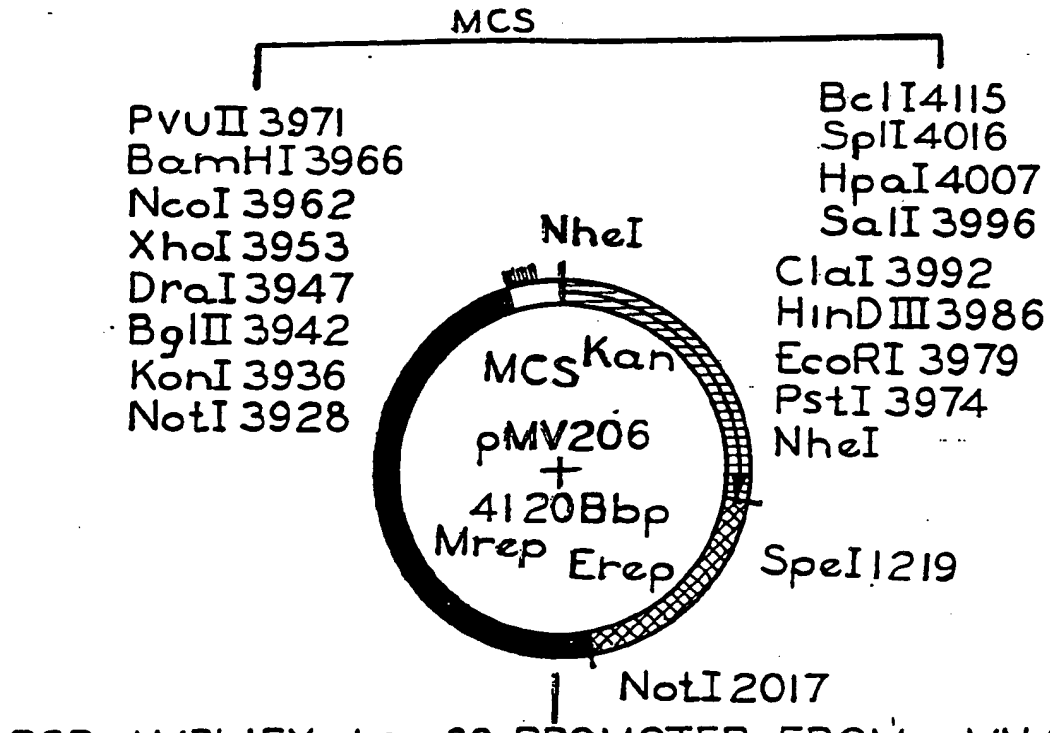
```
   CATCATGGCCGCGGTGATCA
```

FIG. 12Ba

FIG. 12Bb



MATCH WITH FIG. 12Ba



PCR AMPLIFY hsp 60 PROMOTER FROM pMV 261 PRIMERS INCLUDING ADDED XbaI-NheI SITES. DIGEST PCRhsp 60 FRAGMENT WITH XbaI AND NheI. LIGATE INTO XbaI DIGESTED pMV206 AND SCREEN FOR CORRECT ORIENTATION.

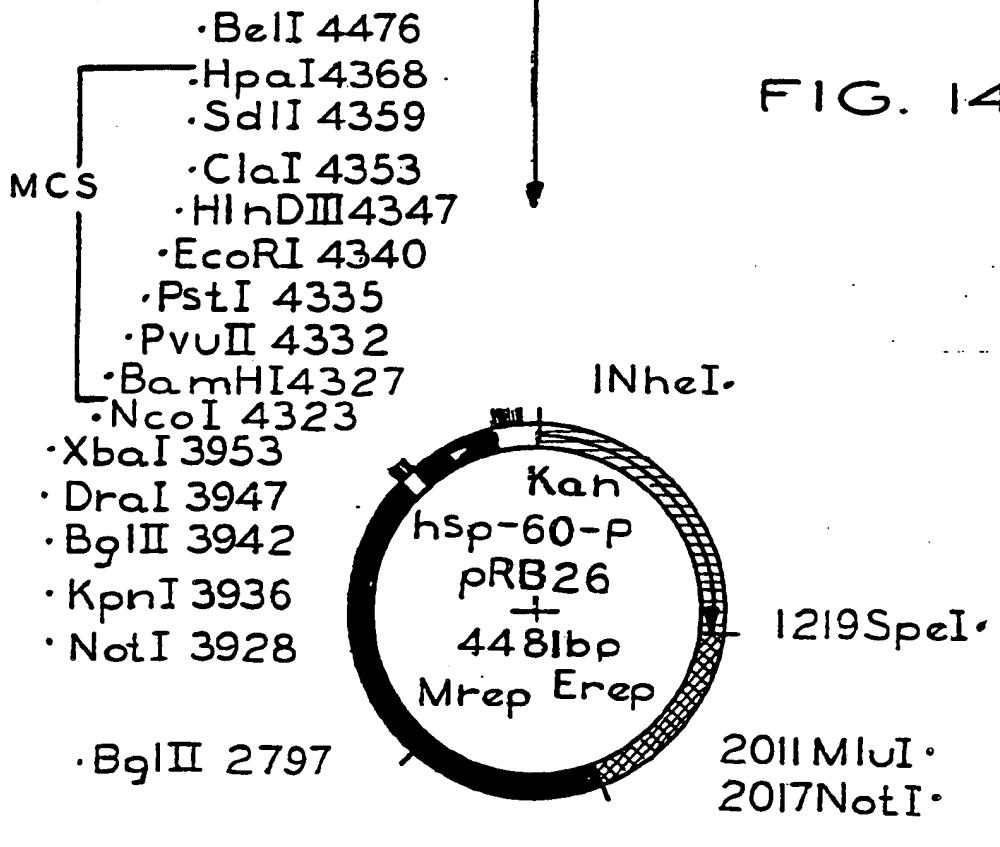


FIG. 14

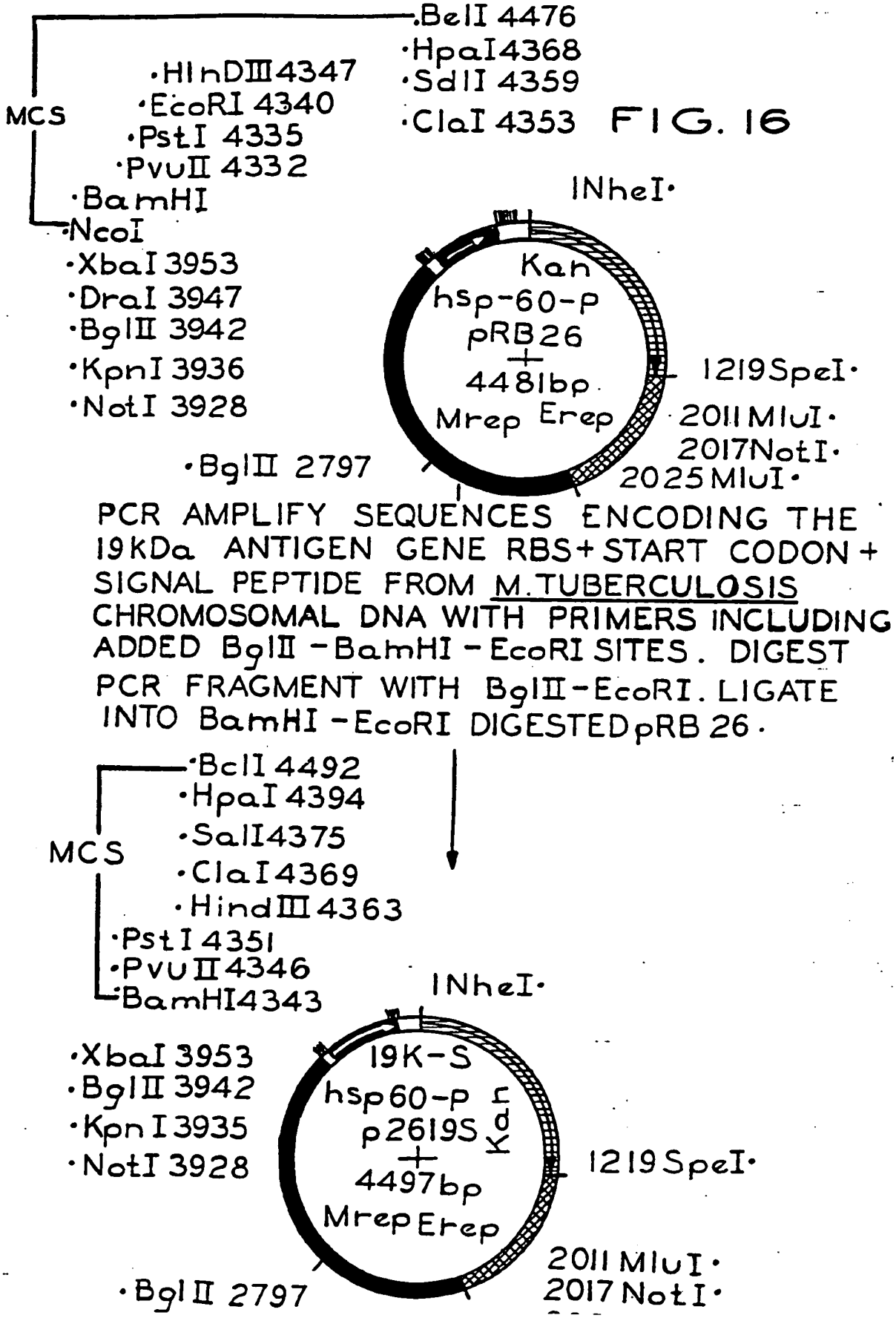

BclI 4476
HpaI 4368
SdlI 4359
ClaI 4353
HInDIII 4347
EcoRI 4340
PstI 4335
PvuII 4332
BamHI
NcoI
MCS
XbaI 3953
DraI 3947
BglII 3942
KpnI 3936
NotI 3928
INheI
Kan
hsp-60-p
pRB26
+
4481bp
Mrep Erep
1219SpeI
2011MluI
2017NotI
2025MluI
BglII 2797
PCR AMPLIFY SEQUENCES ENCODING THE 19KDa ANTIGEN GENE RBS+START CODON+ SIGNAL PEPTIDE FROM M.TUBERCULOSIS CHROMOSOMAL DNA WITH PRIMERS INCLUDING ADDED BglII-BamHI-EcoRI SITES. DIGEST PCR FRAGMENT WITH BglII-EcoRI. LIGATE INTO BamHI-EcoRI DIGESTED pRB 26.
BclI 4492
HpaI 4394
SalI 4375
ClaI 4369
HindIII 4363
PstI 4351
PvuII 4346
BamHI 4343
MCS
XbaI 3953
BglII 3942
KpnI 3935
NotI 3928
INheI
19K-S
hsp60-p
p2619S
+
4497bp
MrepErep
1219SpeI
2011MluI
2017NotI
BglII 2797

FIG. 16

28/63

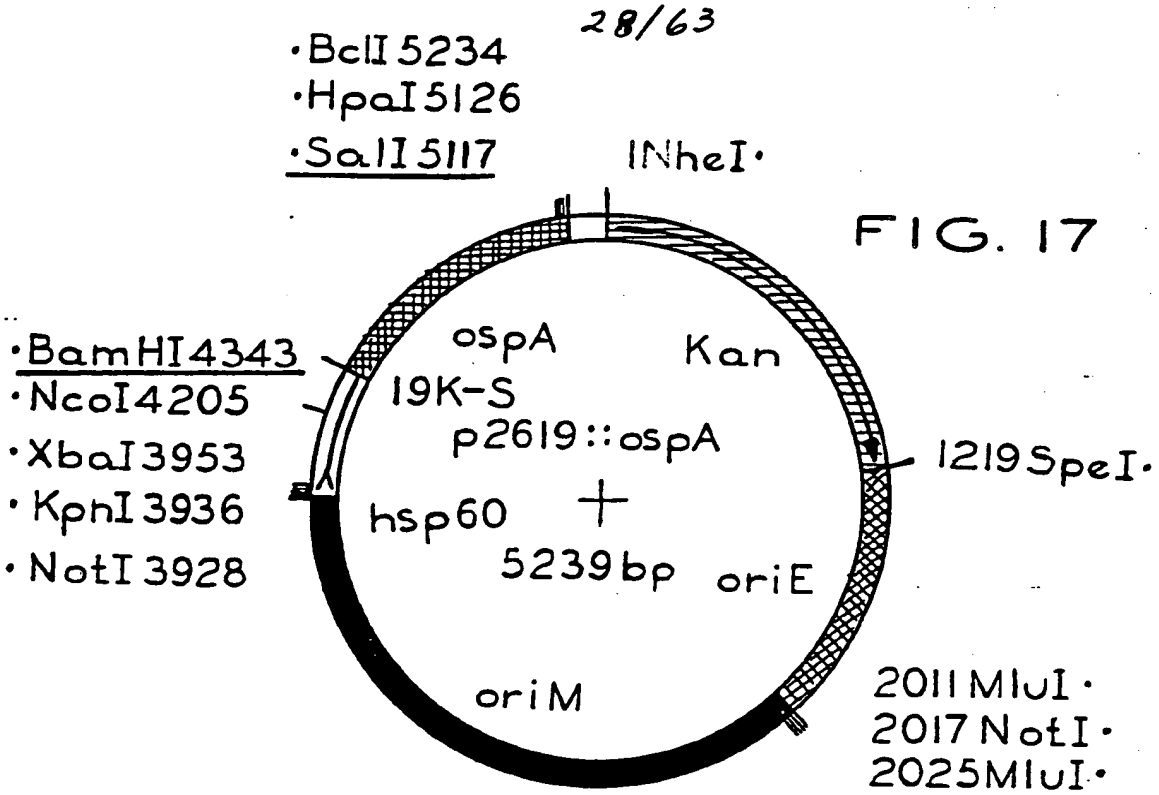


FIG. 17

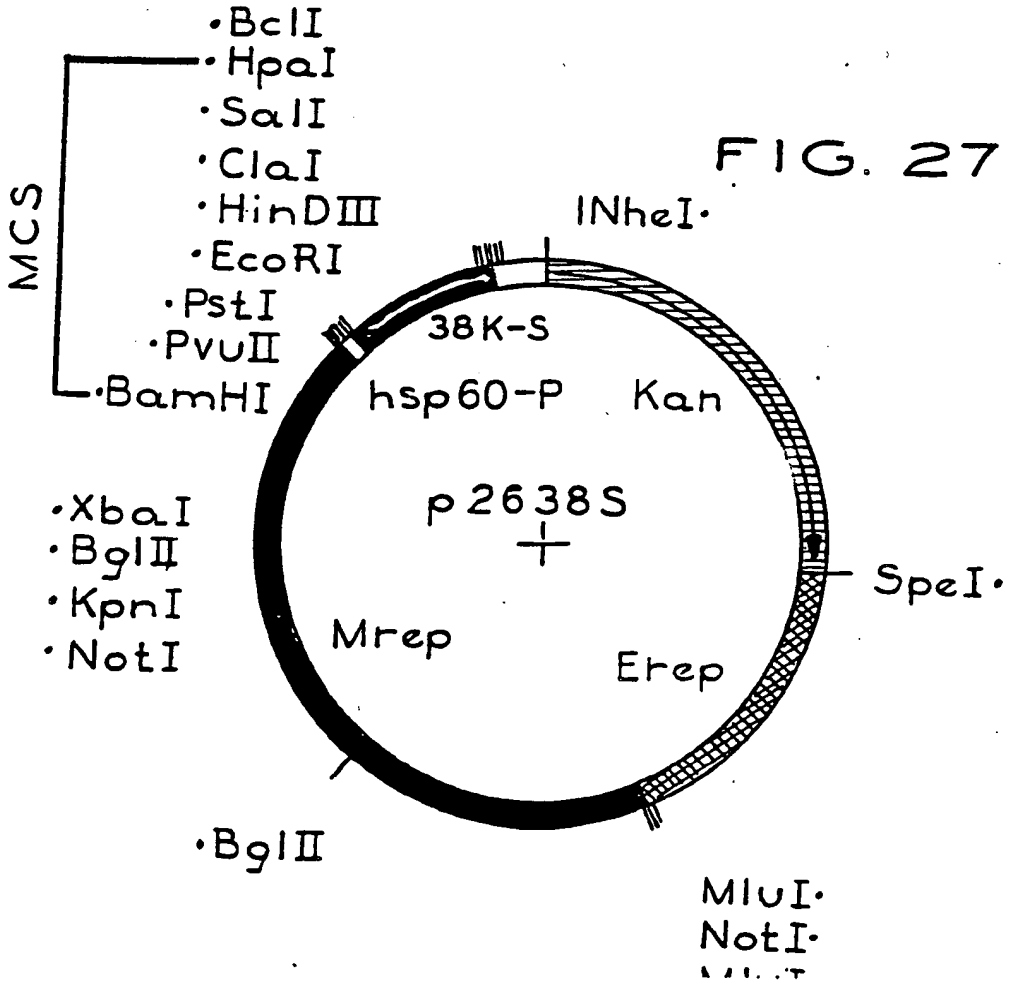


FIG. 27

FIG. 18

```
      1   10      1   20      1   30      1   40      1   50      1   60
  1 TCTAGACAAG GTCGAACGAG GGGCATGACC CGGTGCGGGG CTTCTTGCAC TCGGCATAGG 60
 61 CGAGTGCTAA GAATAACGTT GGCACTCGCG ACCGGTGAGT CGTAGGTCGG GACGGTGAGG 120
 21 CCAGGCCCGT CGTCGCAGCG AGTGGCAGCG AGGACAACTT GAGCCGTCCG TCGCGGGCAC 180
 81 TGCGCCCGGC CAGCGTAAGT AGCGGGGTTG CCGTCACCCG GTGACCCCCG GTTTCATCCC 240
 41 CGATCCGGAG GAATCACTTC GCCATGG                                     267
      1   10      1   20      1   30      1   40      1   50      1   60
```


FIG. 26

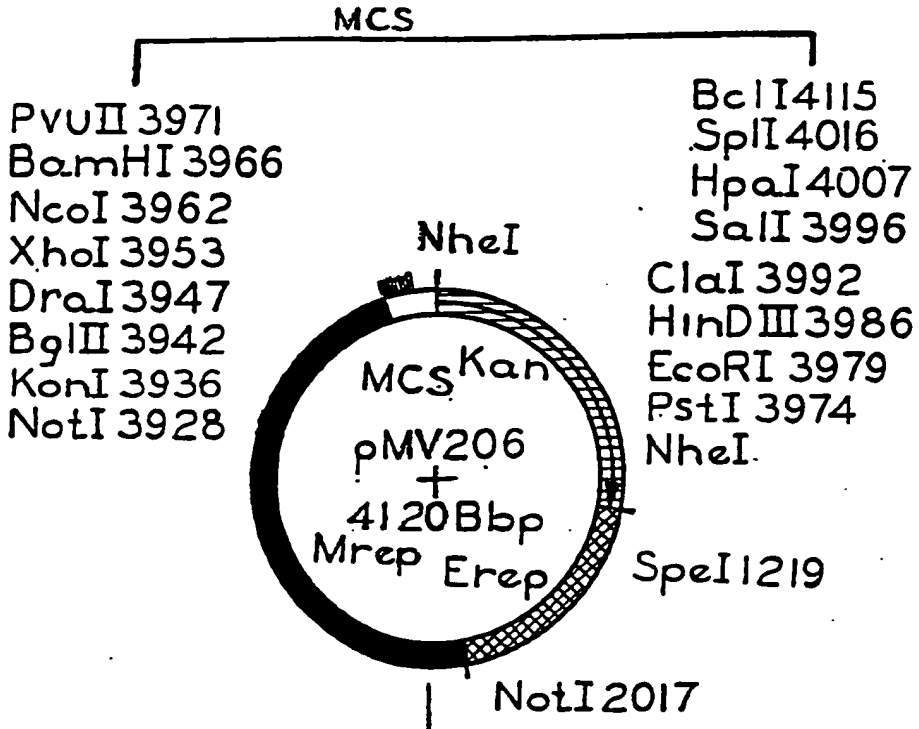
```
      1   10      1   20      1   30      1   40      1   50      1   60
  1 AGATCTGGAC GTCAAGGACG CCAAGCGCGG AAATTGAAGA GCACAGAAAG GTATGGCGTG 60
  1 AAAATTCGTT TGCATACGCT GTTGGCCGTG TTGACCGCTG CGCCGCTGCT GCTAGCAGCG 120
  1 GCGGGCTGTG GCTCGAAACC ACCGAGCGGT TCGCCTGAAA CGGGCGCCGG CGCCGGTACT 180
  1 GTCGCGACTA CGGATCCAGC TGCAGAATTC                                  210
      1   10      1   20      1   30      1   40      1   50      1   60
```

FIG. 13

```
          1   10        1   20        1   30        1   40        1   50        1   60
  1 TCTAGACAAG GTCGAACGAG GGGCATGACC CGGTGCGGGG CTTCTTGCAC TCGGCATAGG 60
 61 CGAGTGCTAA GAATAACGTT GGCACTCGCG ACCGGTGAGT CGTAGGTCGG GACGGTGAGG 120
121 CCAGGCCCGT CGTCGCAGCG AGTGGCAGCG AGGACAACTT GAGCCGTCCG TCGGGGGCAC 180
181 TGCGCCCGGC CAGCGTAAGT AGCGGGGTTG CCGTCACCCG GTGACCCCCG GTTTCATCCC 240
241 CGATCGCTAG C                                                      251
          1   10        1   20        1   30        1   40        1   50        1   60
```

FIG. 15

```
          1   10        1   20        1   30        1   40        1   50        1   60
  1 AGATCTGTCC TCAATGCCGA TGGACCGCTA CGACAGGCAA AGGAGCACAG GGTGAACCGT 60
 61 GGACTGACGG TCGCGGTAGC CGGAGCCGCC ATTCTGGTCG CAGGTCTTTC CGGATGTTCA 120
121 AGCAACAAGT CCACGGATCC AGCTGCAGAA TCC                             153
          1   10        1   20        1   30        1   40        1   50        1   60
```



PCR AMPLIFY SEQUENCES ENCODING THE 19KDa ANTIGEN GENE PROMOTER + RBS + START CODON + SIGNAL PEPTIDE FROM M. TUBERCULOSIS CHROMOSOMAL DNA WITH PRIMERS INCLUDING ADDED XbaI-BamHI SITES. DIGEST PCR FRAGMENT WITH XbaI - BamHI. LIGATE INTO XbaI - BomHI DIGESTED pMV206.

FIG. 19

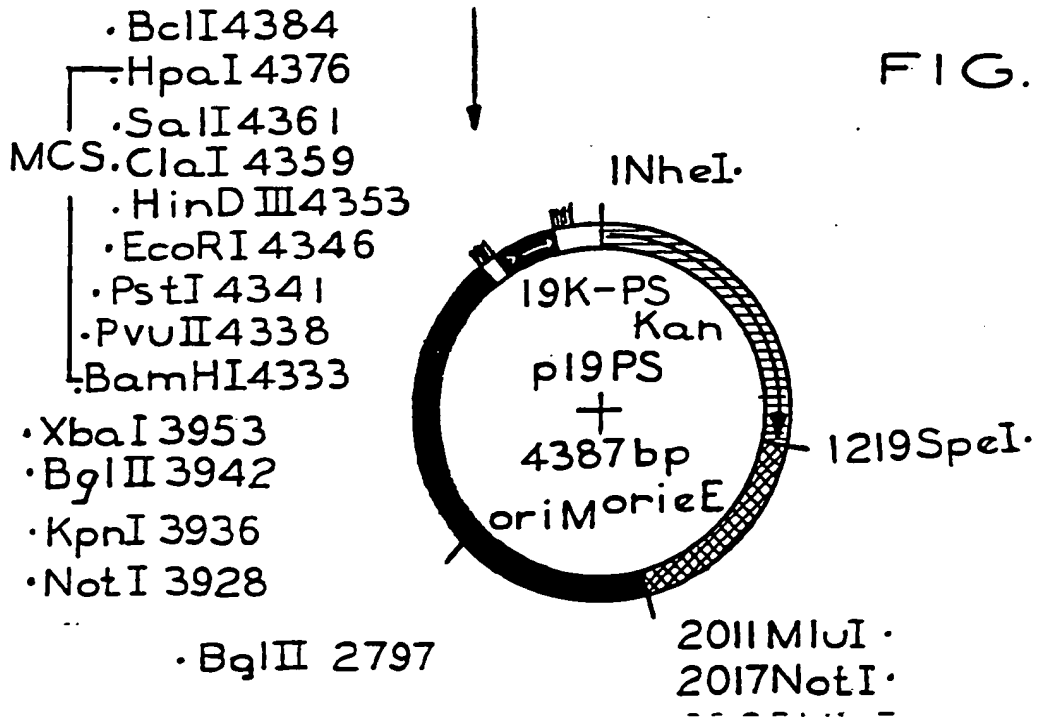


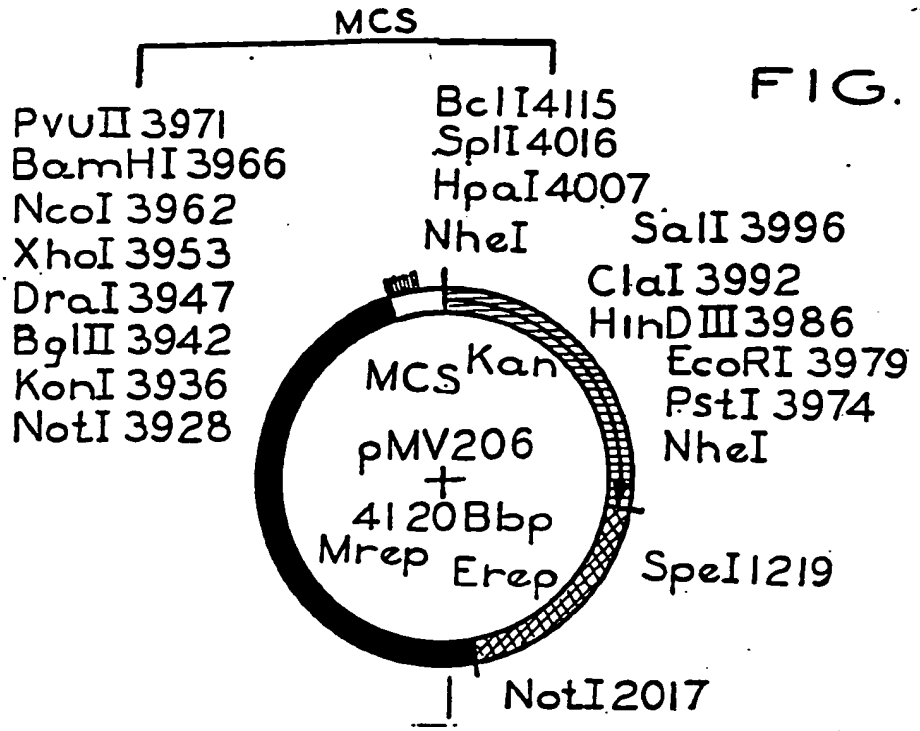
FIG. 20

```
      1   10      1   20      1   30      1   40      1   50      1   60
  1 TCTAGACGGT TTGTGTTCCA TCGGCACTAC ATTGCCACTA CTACGGTGCA CGCCGGTAGA  60
 61 TGCCGTTGGC GAACCACGCT ACCGACCAGA AAGAGAGAAT TTTCCGCCGC ACCTAGACCT 120
121 CGGGCCCTGC TAACGCGCAT ACTGCCGAAG CGGTCCTCAA TGCCGATGGA CCGCTACGAC 180
181 AGGCAAAGGA GCACAGGGTG AAGCGTGGAC TGACGGTCGC GGTAGCCGGA GCCGCCATTC 240
241 TGGTCGCAGG TCTTTCCGGA TGTTCAAGCA ACAAGTCCAC GGATCC                 286
      1   10      1   20      1   30      1   40      1   50      1   60
```

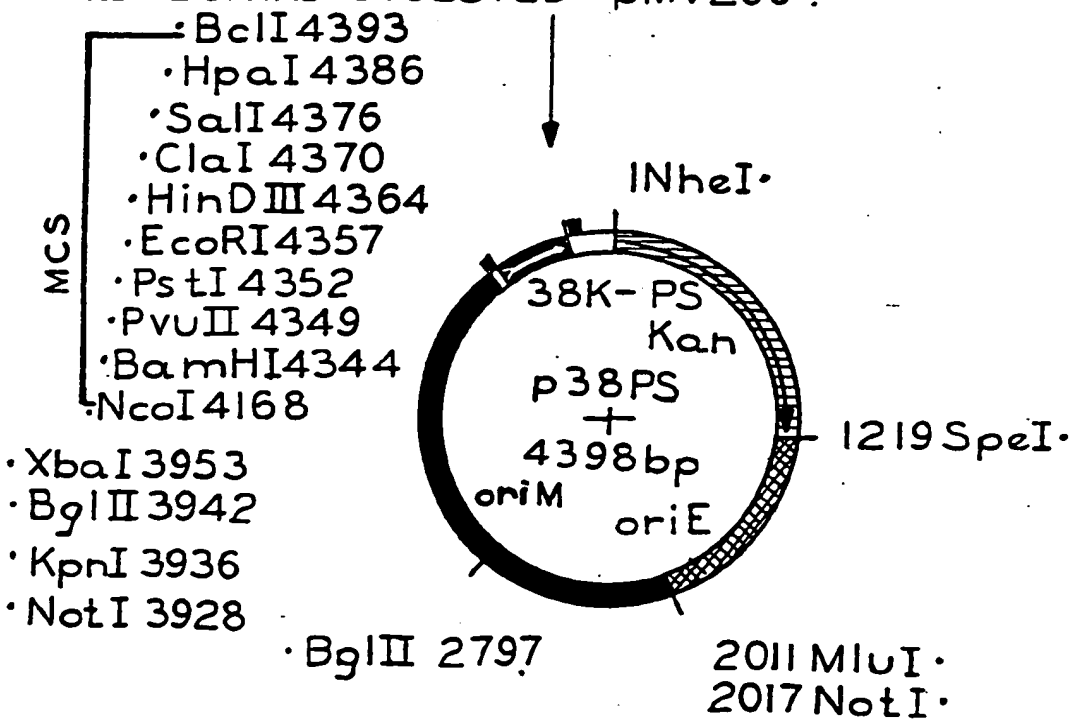

FIG. 24

```
      1   10      1   20      1   30      1   40      1   50      1   60
  1 TCTAGATGTT CTTCGACGGC AGGCTGGTGG AGGAAGGGCC CACCGAACAG CTATTCTCCT  60
 61 CGCCGAAGCA TGCGGAAACC GCCCGATACG TCGCCGGACT GTCGGGGGAC GTCAAGGACG 120
121 CCAAGCGCGG AAATTGAAGA GCACACAAAG GTATGGCGTG AAAATTCGTT TGCATACGCT 180
181 GTTGGCCGTG TTGACCGCTG CGCCGCTGCT GCTAGCAGCG GCGGGCTGTG GCTCGAAACC 240
241 ACCGAGCGGT TCGCCTGAAA CGGGCGCCGG CGCCGGTACT GTCGCGACTA CGGATCC     297
      1   10      1   20      1   30      1   40      1   50      1   60
```

FIG. 21



PCR AMPLIFY SEQUENCES ENCODING THE 38kDa ANTIGEN GENE PROMOTER + RBS + START CODON + SIGNAL PEPTIDE FROM M. TUBERCULOSIS CHROMOSOMAL DNA WITH PRIMERS INCLUDING ADDED XbaI-BamHI SITES. DIGEST PCR FRAGMENT WITH XbaI-BamHI. LIGATE INTO XbaI-BomHI DIGESTED pMV206.



HSP 61 Cassette

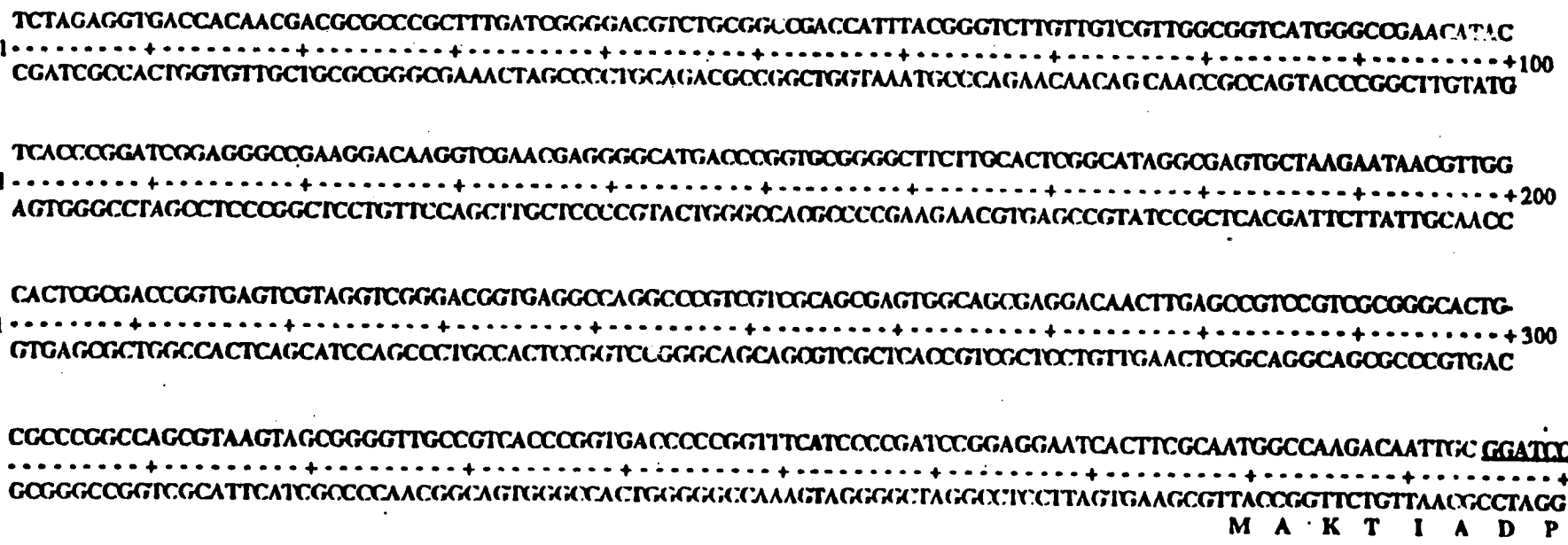


FIG. 22

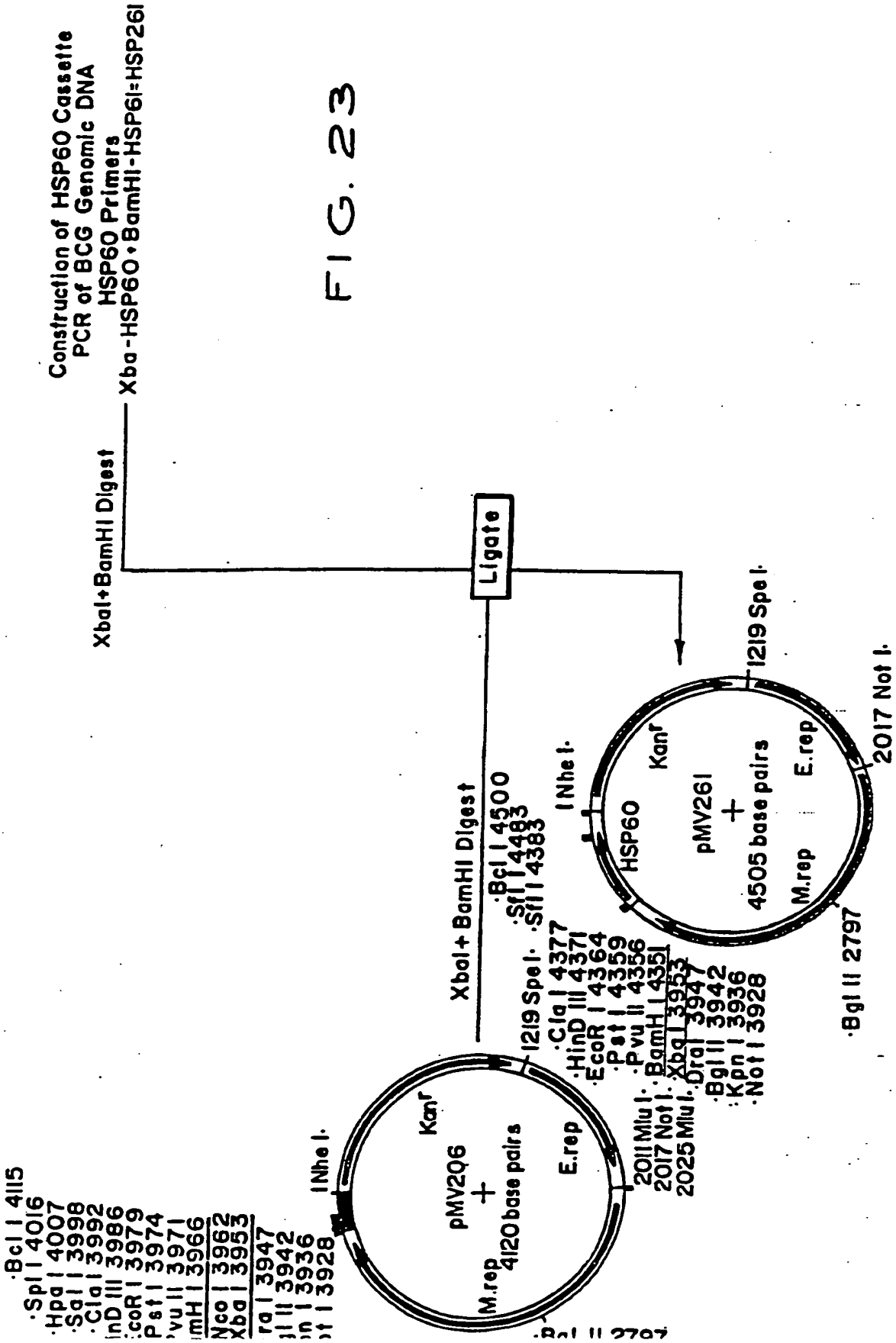

Construction of HSP60 Cassette
PCR of BCG Genomic DNA
HSP60 Primers
Xba-HSP60+BamHI-HSP61=HSP261
XbaI+BamHI Digest
Ligate
XbaI+BamHI Digest
pMV206
+
4120 base pairs
Kanr
E.rep
M.rep
pMV261
+
4505 base pairs
HSP60
Kanr
E.rep
M.rep
INheI
1219 SpeI
2017 NotI
BglII 2797
BclI 4500
SfiI 4483
SfiI 4383
ClaI 4377
HinDIII 4371
EcoRI 4364
PstI 4359
PvuII 4356
BamHI 4351
XbaI 3953
BglII 3947
KpnI 3942
NotI 3936
SpeI 3928
2011 MluI
2017 NotI
2025 MluI
OraI 3947
BclI 4115
SplI 4016
HpaI 4007
SalI 3998
ClaI 3992
HinDIII 3986
EcoRI 3979
PstI 3974
PvuII 3971
HmH I 3966
NcoI 3962
XbaI 3953
raI 3947
II 3942
n I 3936
t I 3928

FIG. 23

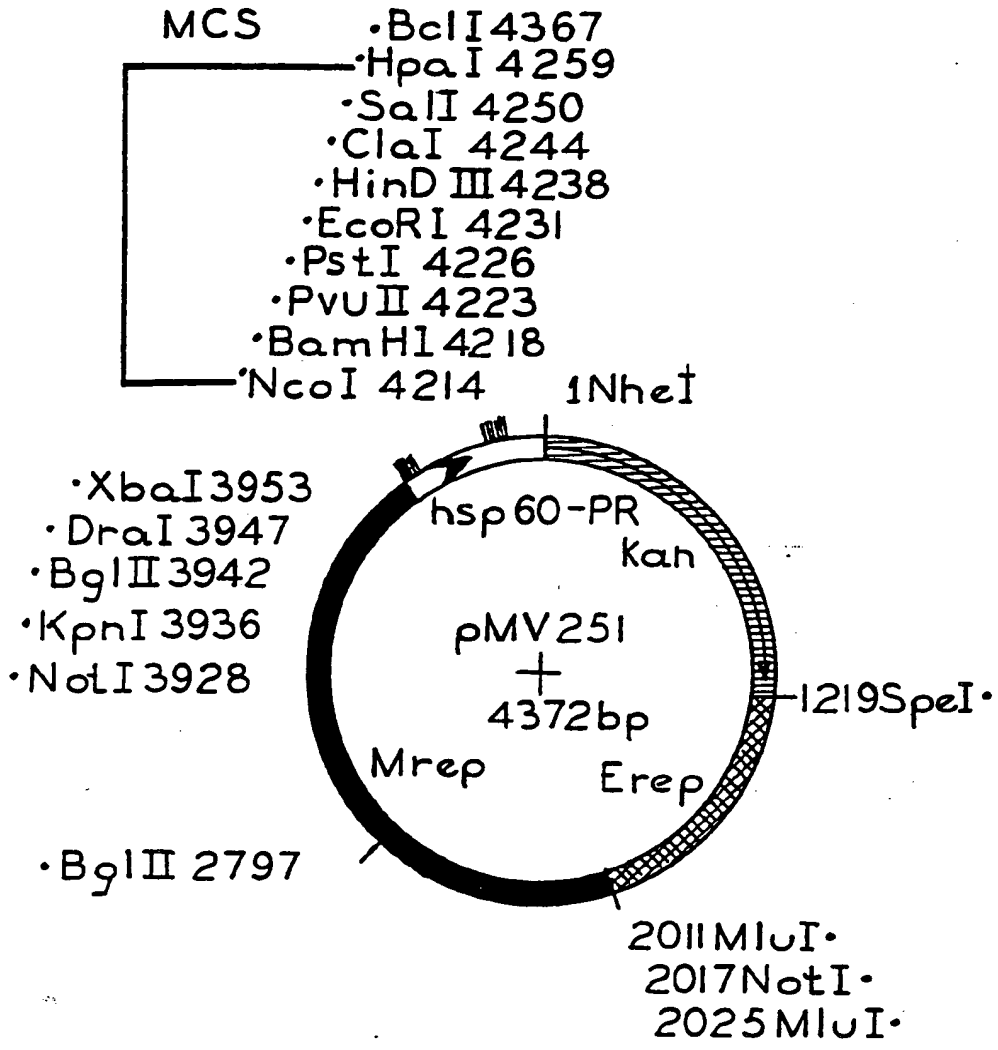

MCS
BclI 4367
HpaI 4259
SalI 4250
ClaI 4244
HinD III 4238
EcoRI 4231
PstI 4226
PvuII 4223
BamHI 4218
NcoI 4214
NheI
XbaI 3953
DraI 3947
BglII 3942
KpnI 3936
NotI 3928
hsp60-PR
Kan
pMV251
+
4372bp
1219SpeI
Mrep
Erep
BglII 2797
2011MluI
2017NotI
2025MluI

FIG. 25

FIG. 28

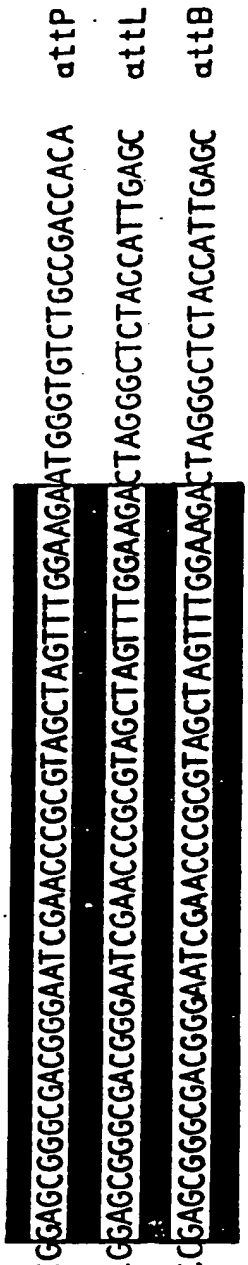
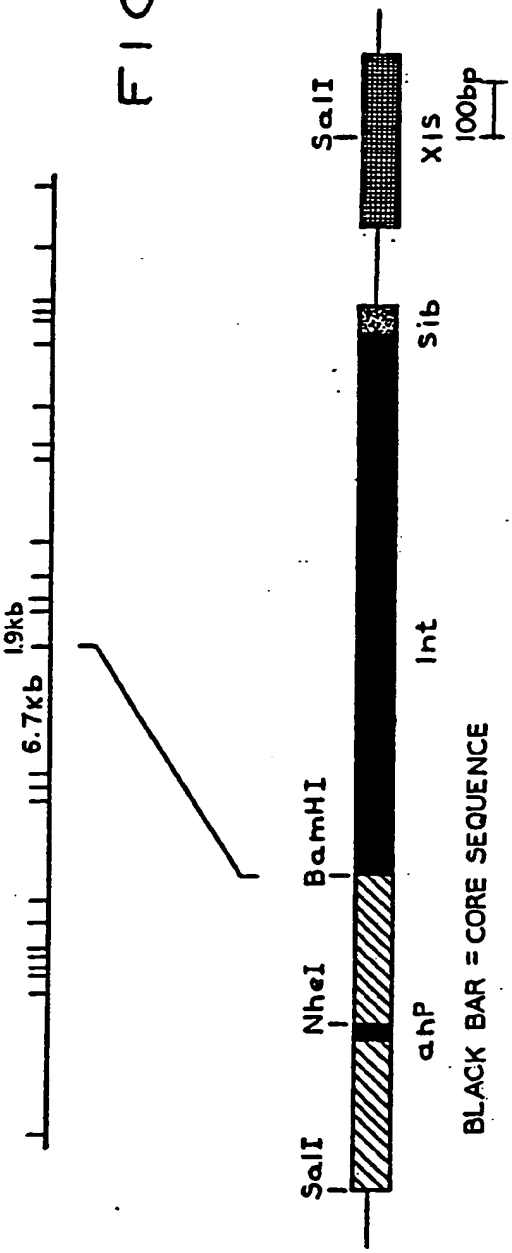

AAAACACCCTCTGACCAGCGGAGCGGGCGACGGGAATCGAACCCGCGTAGCTAGTTTGGAAGAATGGGTGTCTGCCGACCACA attP
AAAACACCCTCTGACCAGCGGAGCGGGCGACGGGAATCGAACCCGCGTAGCTAGTTTGGAAGACTAGGGCTCTACCATTGAGC attL
CGCACGTGGCGGTCCCTACCGAGCGGGCGACGGGAATCGAACCCGCGTAGCTAGTTTGGAAGACTAGGGCTCTACCATTGAGC attB

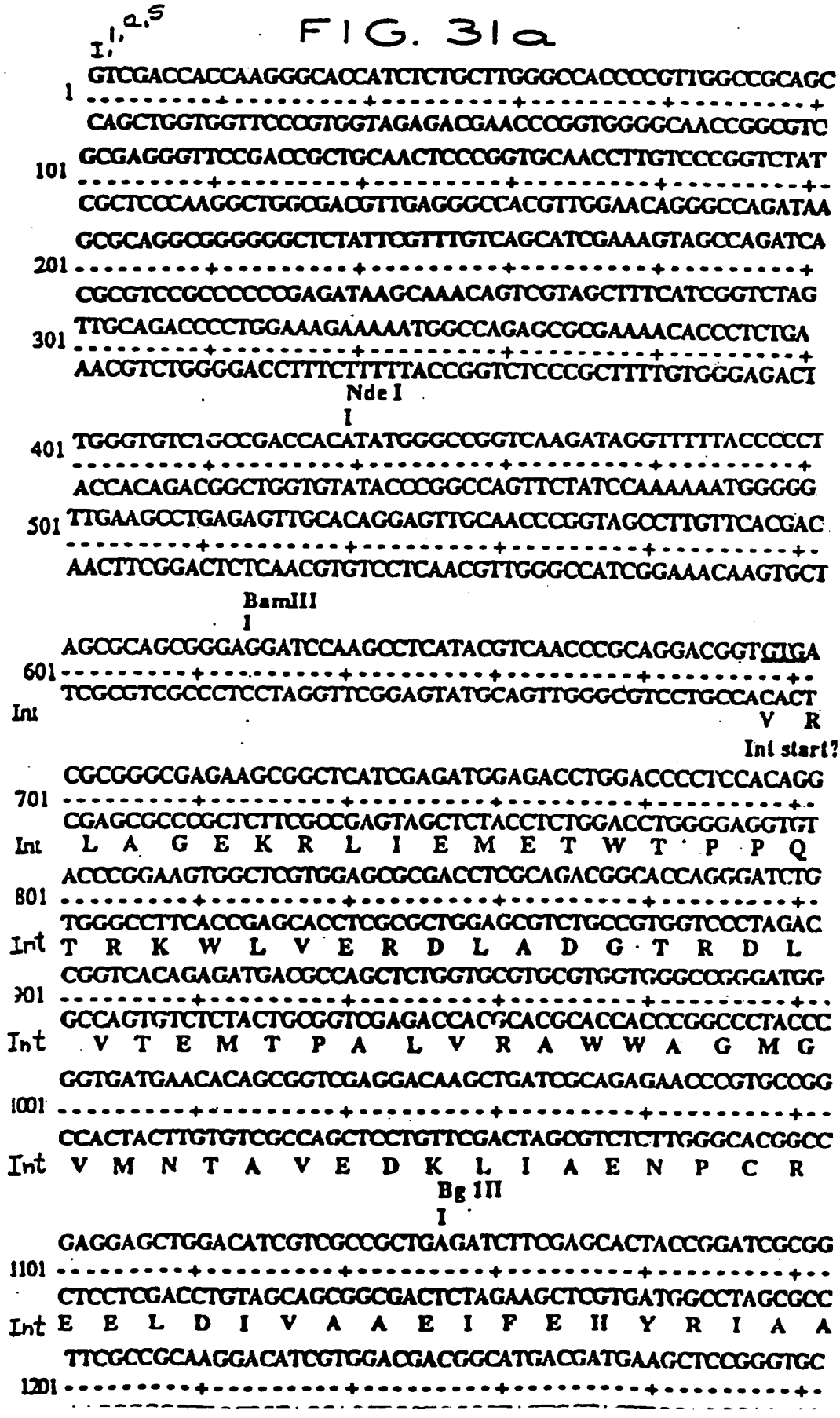
FIG. 30


L5 BAM HI SITES
5kb
1.9kb
6.7kb
SalI
NheI
BamHI
ahP
Int
Sib
XIS
SalI
100bp
BLACK BAR = CORE SEQUENCE

	DOMAIN 1	DOMAIN 2
(λ)	RLAMELAVVTGQRVGDLCEMKWSDIVDG	HELRSLSA-RLYEKQ-ISDKFAQHLLGHKS-DTMASQYR-
(HK22)	RLAMDLAVVTGQRVGDLCRMKWSDIVDG	HELRSLSA-RLYRNQ-IGDKFAQRLLGHKS-DSMAARYRD
(φ 80)	VFLVKFIMLTGCRTAEIRLSERSWFRLD	HDMRRTIATNLSELG-CPPHVIEKLLGHQM-VGVMAHYN-
(P2)	KKIAILCLSTGARWGEAARLKAENIIHN	HALRHSFATHFMING-GSIITLQRILGHTR-IEQTMVYAH
(P4)	MIAVKLSLLTFVRSSELRFARWDEFDFD	HGFRTMARGALGESGLWSDDAIERQSLHSERNNVRAAYIH
(P22)	KSVVEFALSTGLRRSNIINLEWQQIDMQ	HDLRHTWASWLVQAG-VPISVLQEMGGWES-IEMVRRYAH
(186)	ETVVRICLATGARWSEAESLRKSQLAKY	HVLRHTFASHFMMNG-GNILVLQRVLGHTD-IKMTMRYAH
(HP1)	GLIVRICLATGARWSEAETLTQSQVMPY	HVLRHTFASHFMMNG-GNILVLKEILGHST-IEMTMRYAH
(L54a)	AGAVEVQALTGMRIGELLALQVKDVDLK	HTLRHTHISLLAEMN-ISLKAIMKRVGHRDEKTTIKVYTH
(P1)	TAGVEKALSLGVTKLVERWISVSGVADD	HSARVGAARDMARAG-VSIPEIMQAGGWTN-VNIVMNYIR
rot. (F)	KMLLATLWNTGARINEALALTRGDFSLA	HTFRHSYAMHMLYAG-IPLKVLQSLMGHKS-ISSTEVYTK
B	YCLTLLCFIHGFRASEICRLRISDIDLK	HMLRHSCGFALANMG-IDTRLIQDYLGHRN-IRHTVRYTA
E	YCLILLAYRHGMRISELLDLHYQDLDLN	HMLRHACGYELAERG-ADTRLIQDYLGHRN-IRHTVRYTA
603 ORF3	RLFAQLLYGTCMRISEGLQLRVKDLDFD	HTLRHSFATALLRSG-YDIRTVQDLLGHSD-VSTTMIYTH
54 TnpA	KLILMLMYEGGLRIGEVLSLRLEDIVTW	HMLRHTHATQLIREG-VDVAFVQKRLGHAHVQTTLNTYVH
54 TnpB	ATMTMIVQECGMRISELCTLKKGCLLED	HAFRHTVGTRMINNG-MPQHIVQKFLGHES-PEMTSRYAH
430 TnpI	YAIATLLAYTGVRISEALSIKMNDFNLQ	HQLRHFFCTNAIEKG-FSIHEVANQAGHSN-IHTTLLYT-
	YVIFHLALETAMRQQEILALRWEHIDLR	HDLRHEAISRFFELGSLNVMEIAAISGHRS-MNMLKRYTH
945 ORF2	YDEILILLKTGLRISEFGGLTLPDLDFE	HIGRHLMTSFLSMKGLTELTNVVGNWSDKRASAVATTYTH
	----------	HIGRHLMTSFLSMKGLTELTNVVGNWSDKRASAVATTYTH
SENSUS	--Iv-L-I-TGmR-SEI--Lr--di---	H-LRHt-At-L---G---I--IQ-ILgh---I--T--Y-H
	RIAAYILAWTSLRFGELIELRRKDIVDD	HDLRAVGATFAAQAG-ATTKELMARLGHTT-PRMAMKYQM

FIG. 29

FIG. 31a



MATCH WITH FIG. 31b

FIG. 31b

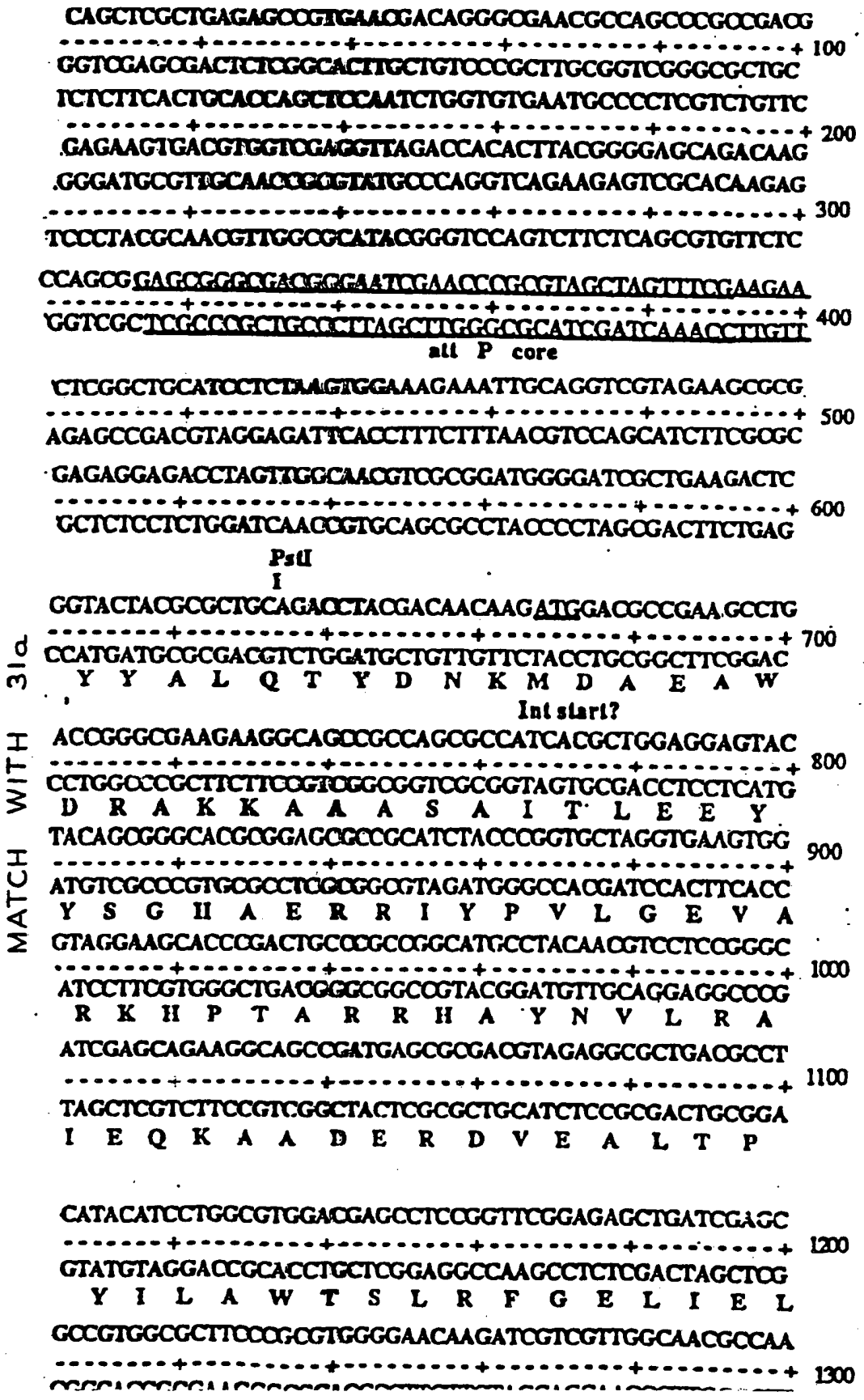
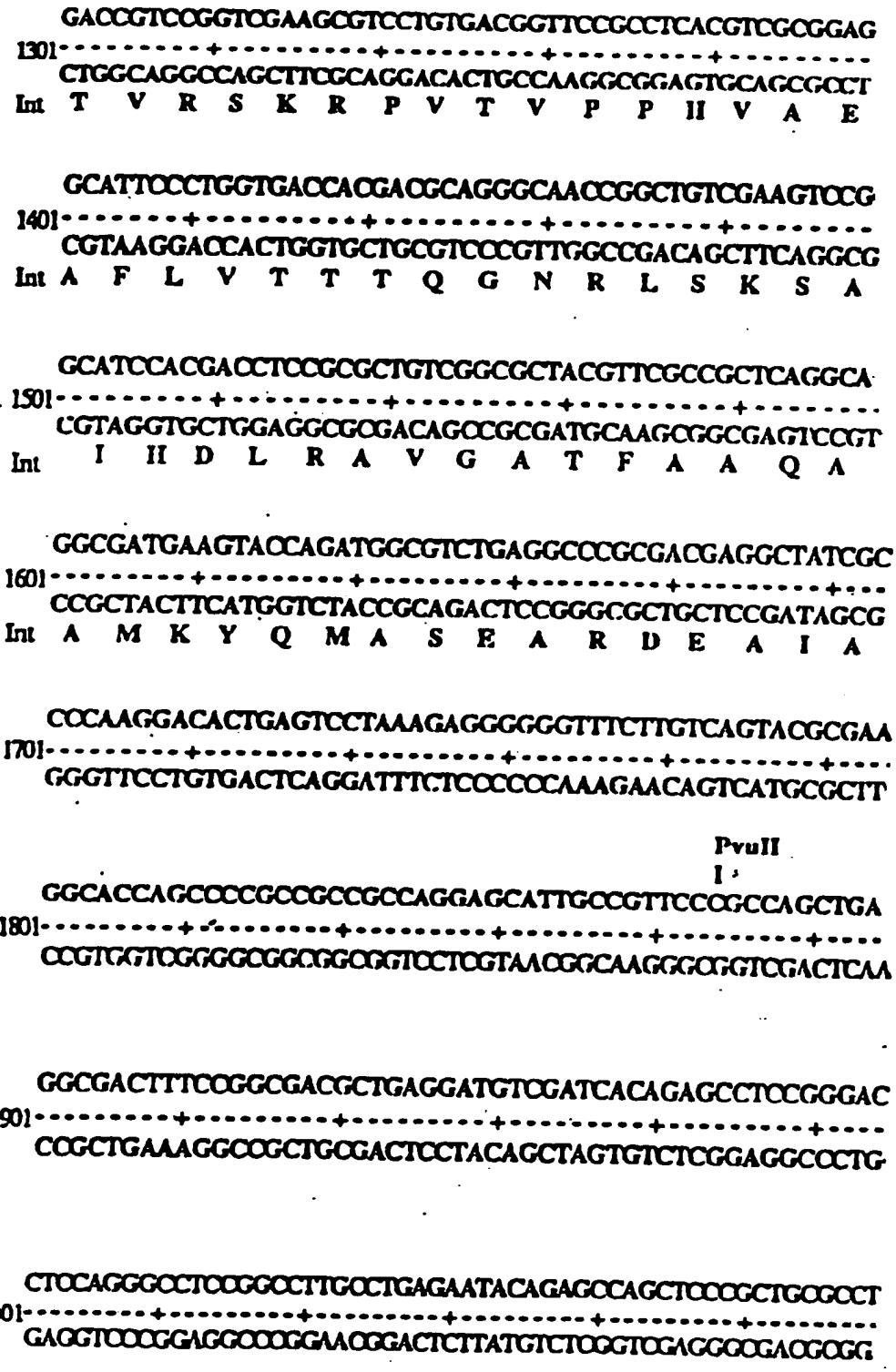
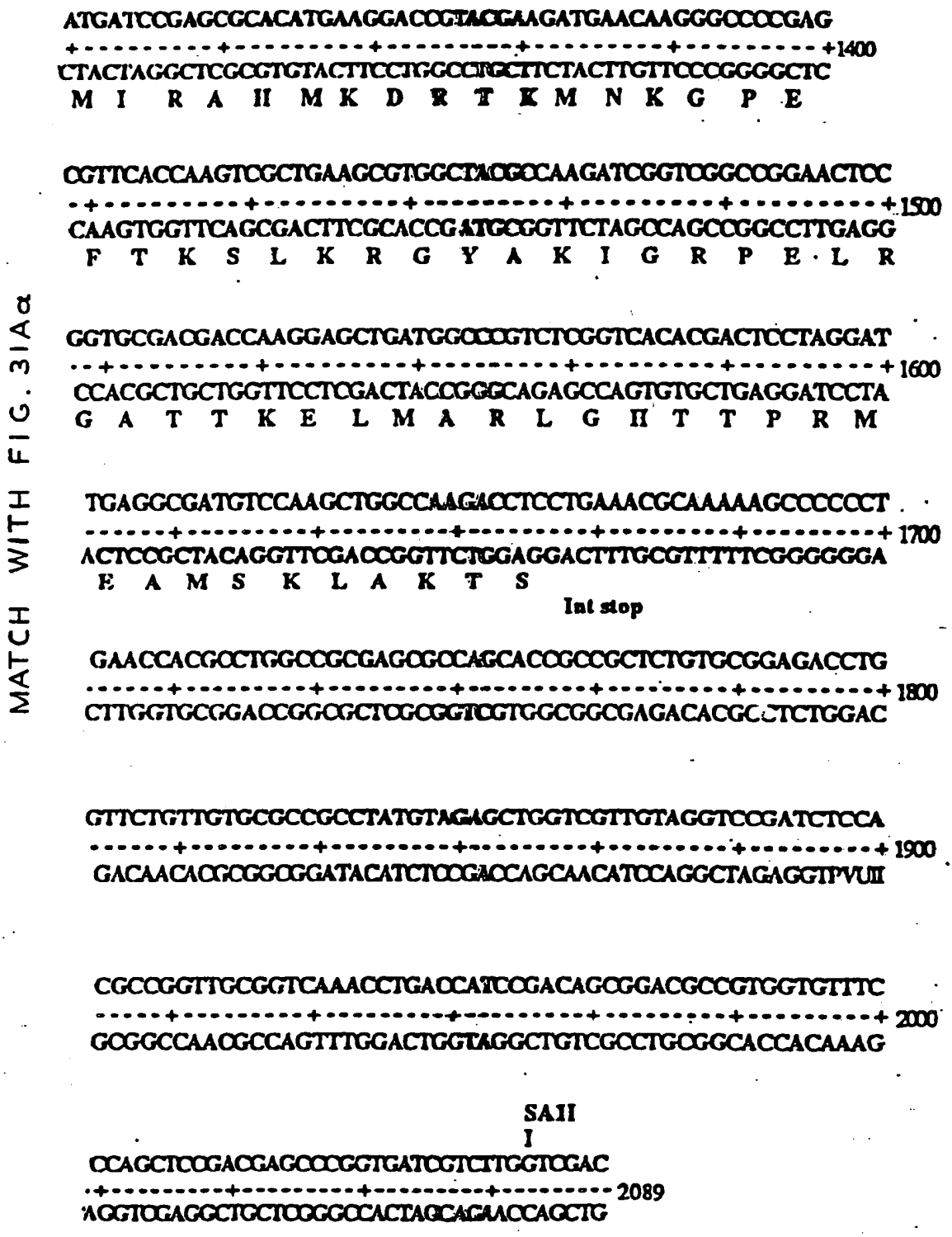

CAGCTCGCTGAGAGCCGTGAACGACAGGGCGAACGCCAGCCCGCCGACG
GGTCGAGCGACTCTCGGCACTTGCTGTCCCGCTTGCGGTCGGGCGCTGC
TCTCTTCACTGCACCAGCTCCAATCTGGTGTGAATGCCCCTCGTCTGTTC
GAGAAGTGACGTGGTCGAGGTTAGACCACACTTACGGGGAGCAGACAAG
GGGATGCGTTGCAACCGGGTATGCCCAGGTCAGAAGAGTCGCACAAGAG
TCCCTACGCAACGTTGGCGCATACGGGTCCAGTCTTCTCAGCGTGTTCTC
CCAGCGGAGCGGGGCGACGGGAATCGAACCCGCGTAGCTAGTTTCGAAGAA
GGTCGCTCGCCGCTGCCCTTAGCTTGGGCGCATCGATCAAACCTTGTT
alt P core
CTCGGCTGCATCCTCTAAGTGGAAAGAAATTGCAGGTCGTAGAAGCGCG
AGAGCCGACGTAGGAGATTCACCTTTCTTTAACGTCCAGCATCTTCGCGC
GAGAGGAGACCTAGTTGGCAACGTCGCGGATGGGGATCGCTGAAGACTC
GCTCTCCTCTGGATCAACCGTGCAGCGCCTACCCCTAGCGACTTCTGAG
PstI
I
GGTACTACGCGCTGCAGACCTACGACAACAAGATGGACGCCGAAGCCTG
CCATGATGCGCGACGTCTGGATGCTGTTGTTCTACCTGCGGCTTCGGAC
Y Y A L Q T Y D N K M D A E A W
Int start?
ACCGGGCGAAGAAGGCAGCCGCCAGCGCCATCACGCTGGAGGAGTAC
CCTGGCCCGCTTCTTCCGTCGGCGGTCGCGGTAGTGCGACCTCCTCATG
D R A K K A A A S A I T L E E Y
TACAGCGGGCACGCGGAGCGCCGCATCTACCCGGTGCTAGGTGAAGTGG
ATGTCGCCCGTGCGCCTCGCGGCGTAGATGGGCCACGATCCACTTCACC
Y S G H A E R R I Y P V L G E V A
GTAGGAAGCACCCGACTGCCCGCGGCATGCCTACAACGTCCTCCGGGC
ATCCTTCGTGGGCTGACGGGCGGCCGTACGGATGTTGCAGGAGGCCCG
R K H P T A R R H A Y N V L R A
ATCGAGCAGAAGGCAGCCGATGAGCGCGACGTAGAGGCGCTGACGCCT
TAGCTCGTCTTCCGTCGGCTACTCGCGCTGCATCTCCGCGACTGCGGA
I E Q K A A D E R D V E A L T P
CATACATCCTGGCGTGGACGAGCCTCCGGTTCGGAGAGCTGATCGAGC
GTATGTAGGACCGCACCTGCTCGGAGGCCAAGCCTCTCGACTAGCTCG
Y I L A W T S L R F G E L I E L
GCCGTGGCGCTTCCCGCGTGGGGAACAAGATCGTCGTTGGCAACGCCAA
31a
MATCH WITH
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300

FIG. 31Aa



MATCH WITH FIG. 31Ab

FIG. 31Ab



MATCH WITH FIG. 31Aα

FIG. 32

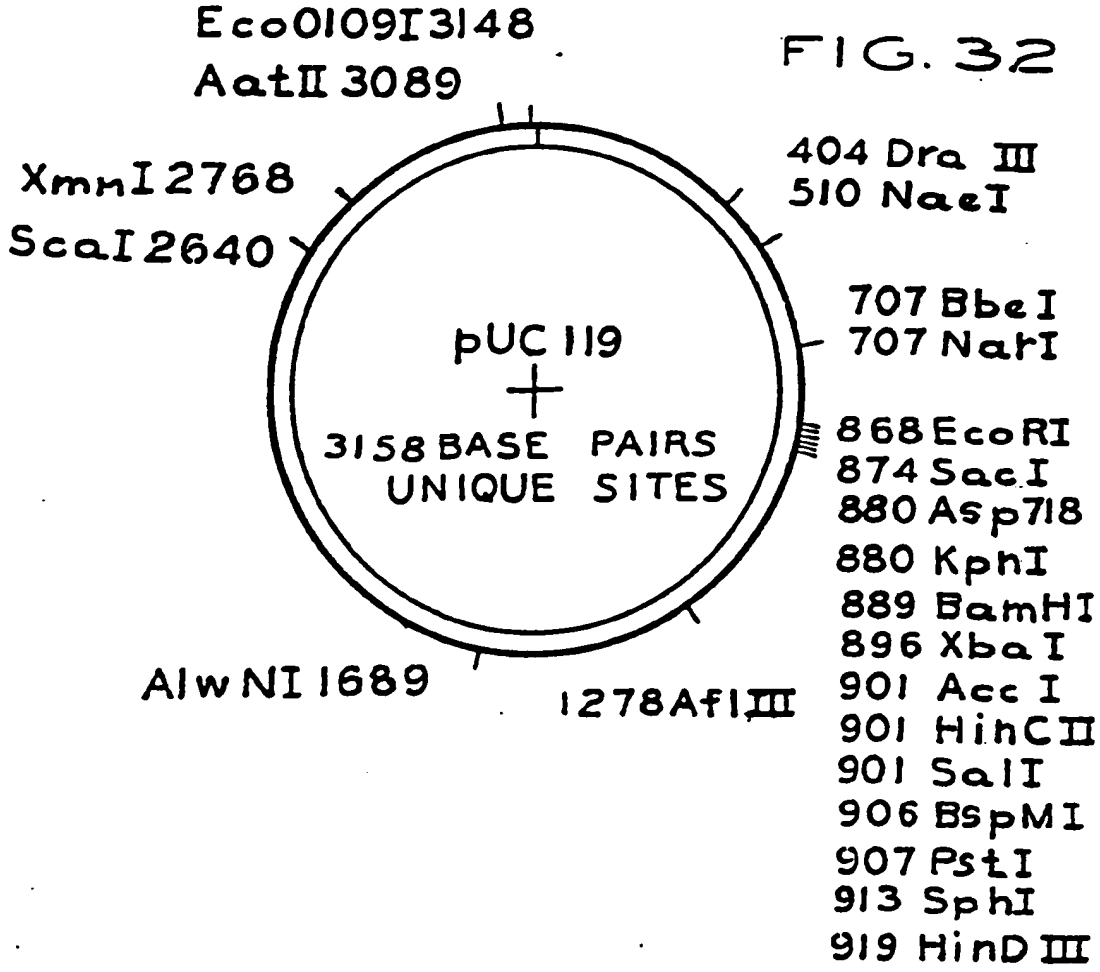

Eco0109I3148
AatII 3089
404 Dra III
510 NaeI
XmnI 2768
ScaI 2640
707 BbeI
707 NarI
pUC 119
+
3158 BASE PAIRS
UNIQUE SITES
868 EcoRI
874 SacI
880 Asp718
880 KpnI
889 BamHI
896 XbaI
901 Acc I
901 HinCII
901 SalI
906 BspMI
907 PstI
913 SphI
919 HinD III
AlwNI 1689
1278 AflIII

FIG. 33

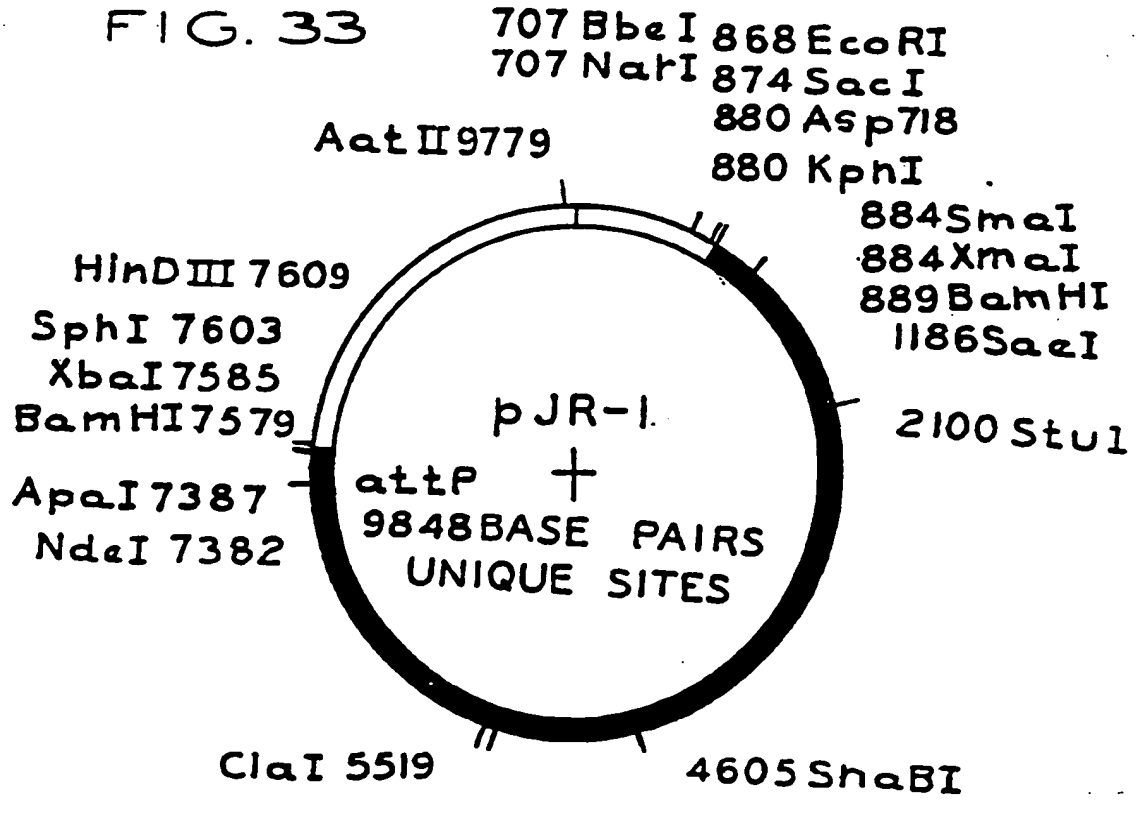
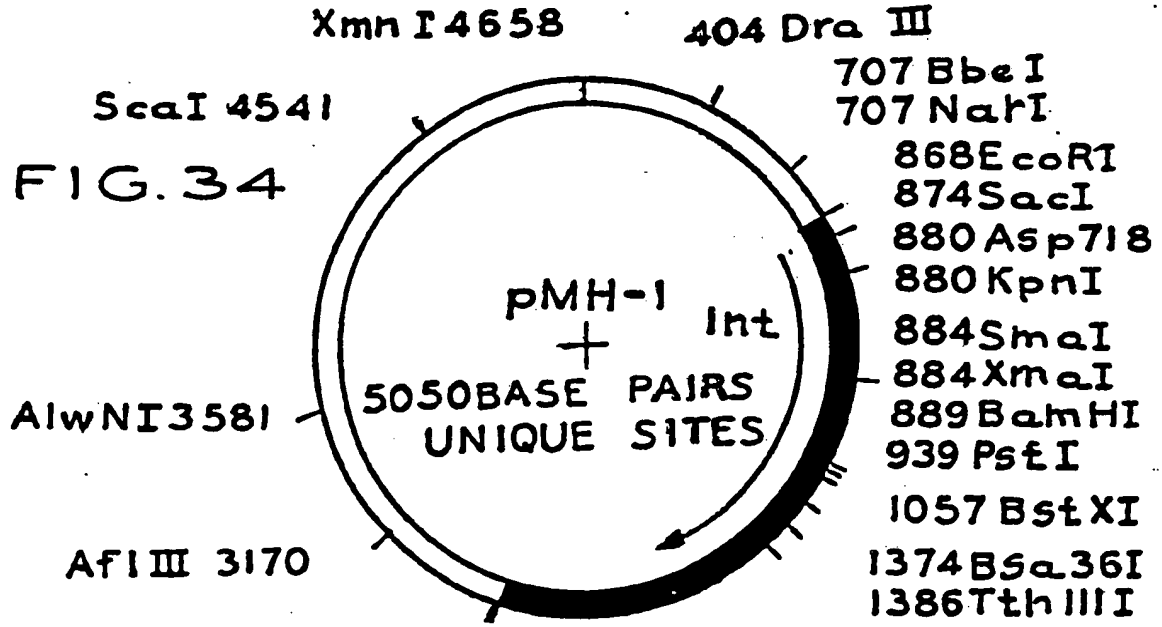
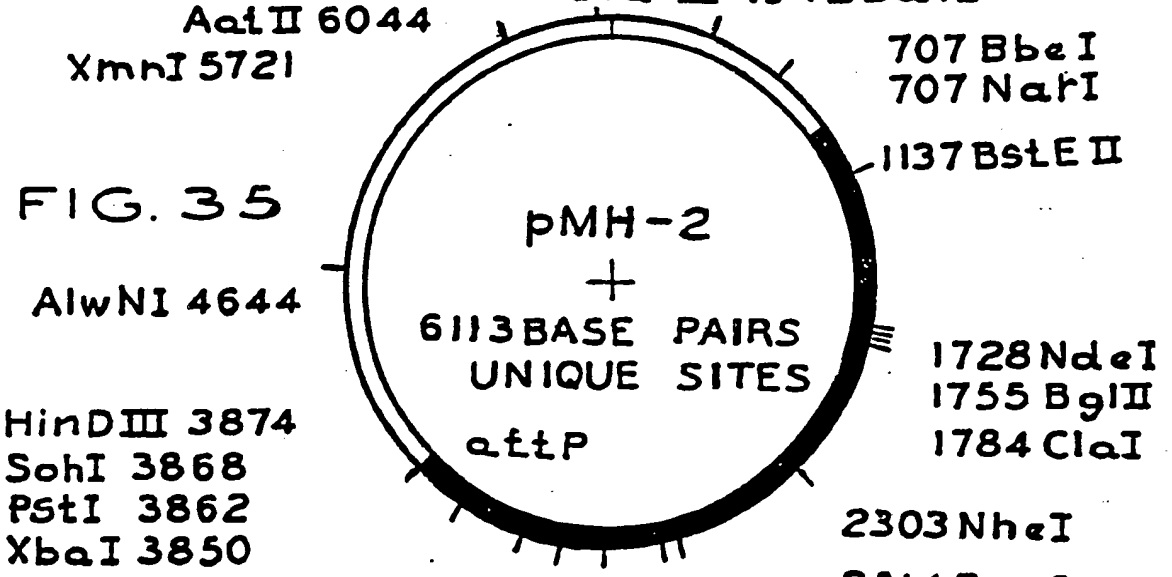

707 BbeI
707 NarI
868 EcoRI
874 SacI
880 Asp718
880 KpnI
AatII 9779
884 SmaI
884 XmaI
889 BamHI
1186 SacI
HinDIII 7609
SphI 7603
XbaI 7585
BamHI 7579
pJR-1
2100 StuI
attP
+
ApaI 7387
NdeI 7382
9848 BASE PAIRS
UNIQUE SITES
ClaI 5519
4605 ShaBI
NdeI 5463

FIG. 34



SHADED PORTION = L5 DNA

FIG. 35



SHADED PORTION = L5 DNA

FIG. 36

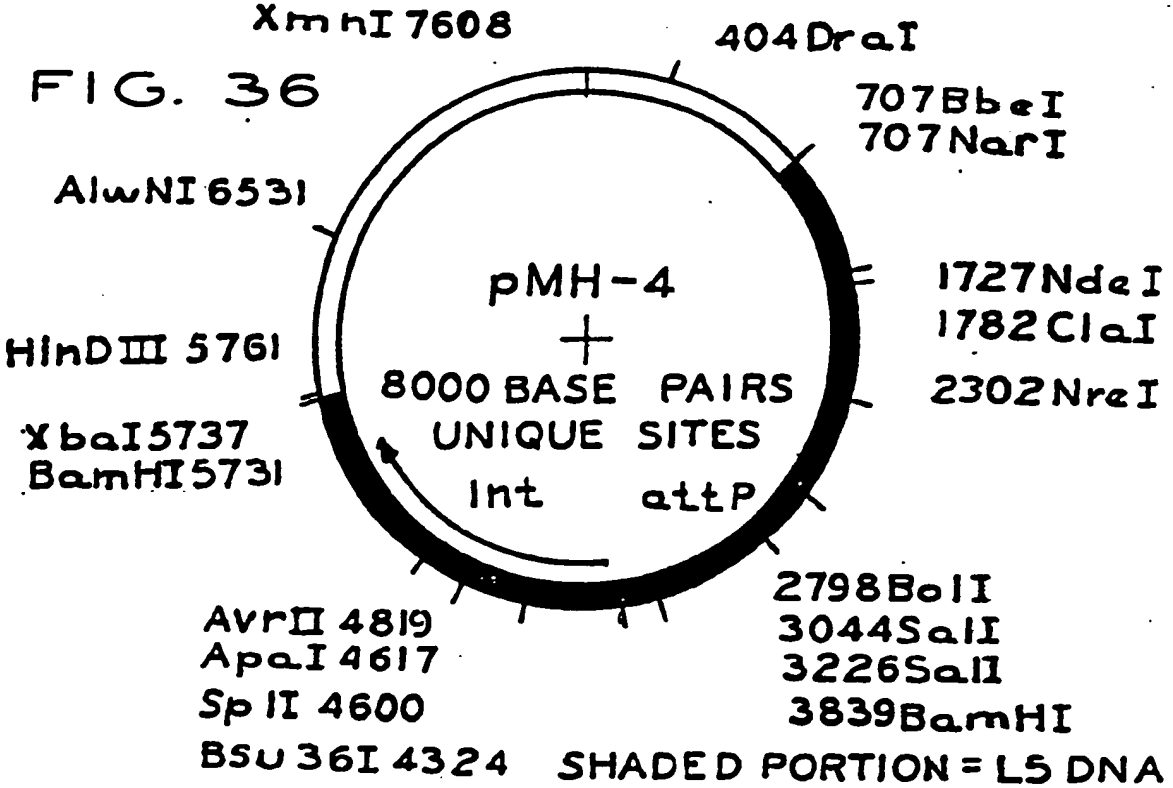

XmnI 7608
404 DraI
707 BbeI
707 NarI
AlwNI 6531
1727 NdeI
1782 ClaI
2302 NreI
pMH-4
+
8000 BASE PAIRS
UNIQUE SITES
Int
attP
HinDIII 5761
XbaI 5737
BamHI 5731
2798 BolI
3044 SalI
3226 SalI
3839 BamHI
AvrII 4819
ApaI 4617
SpII 4600
Bsu 36I 4324
SHADED PORTION = L5 DNA
404 Dra III

FIG. 37

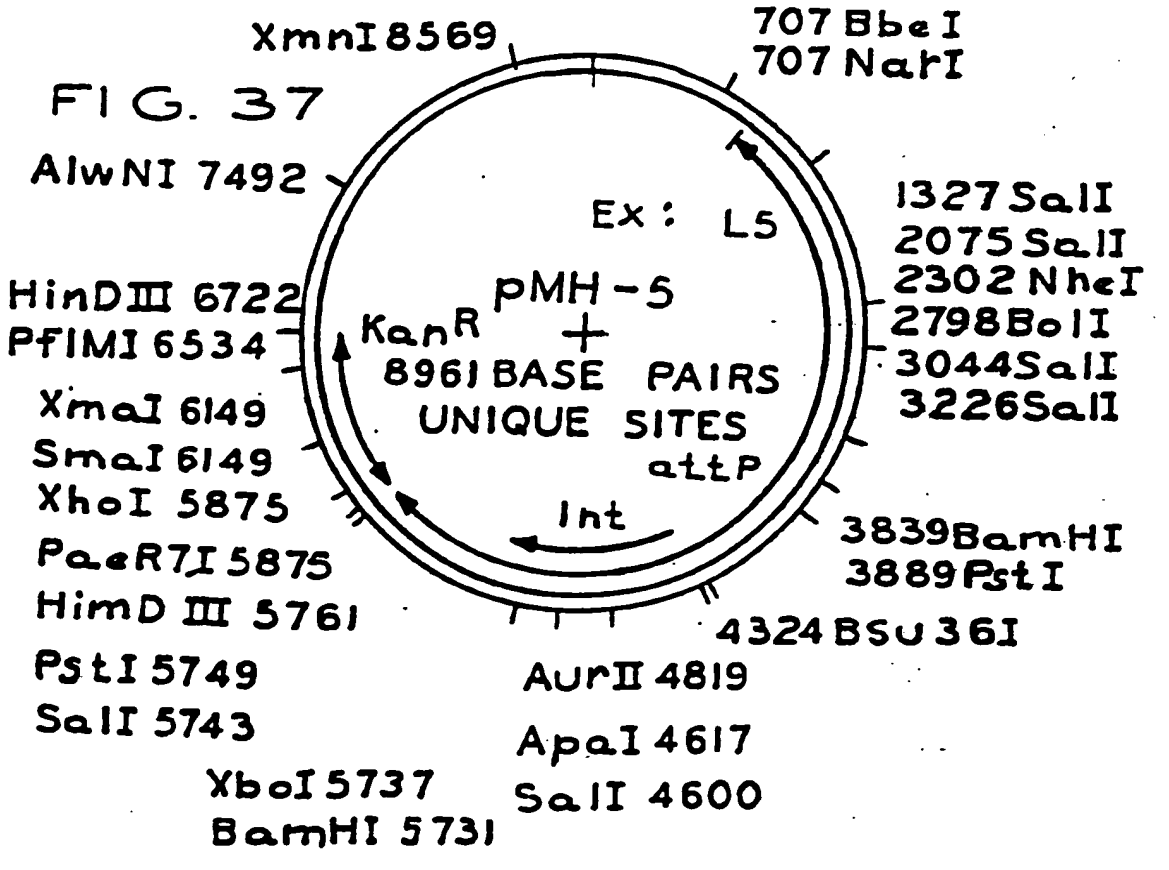
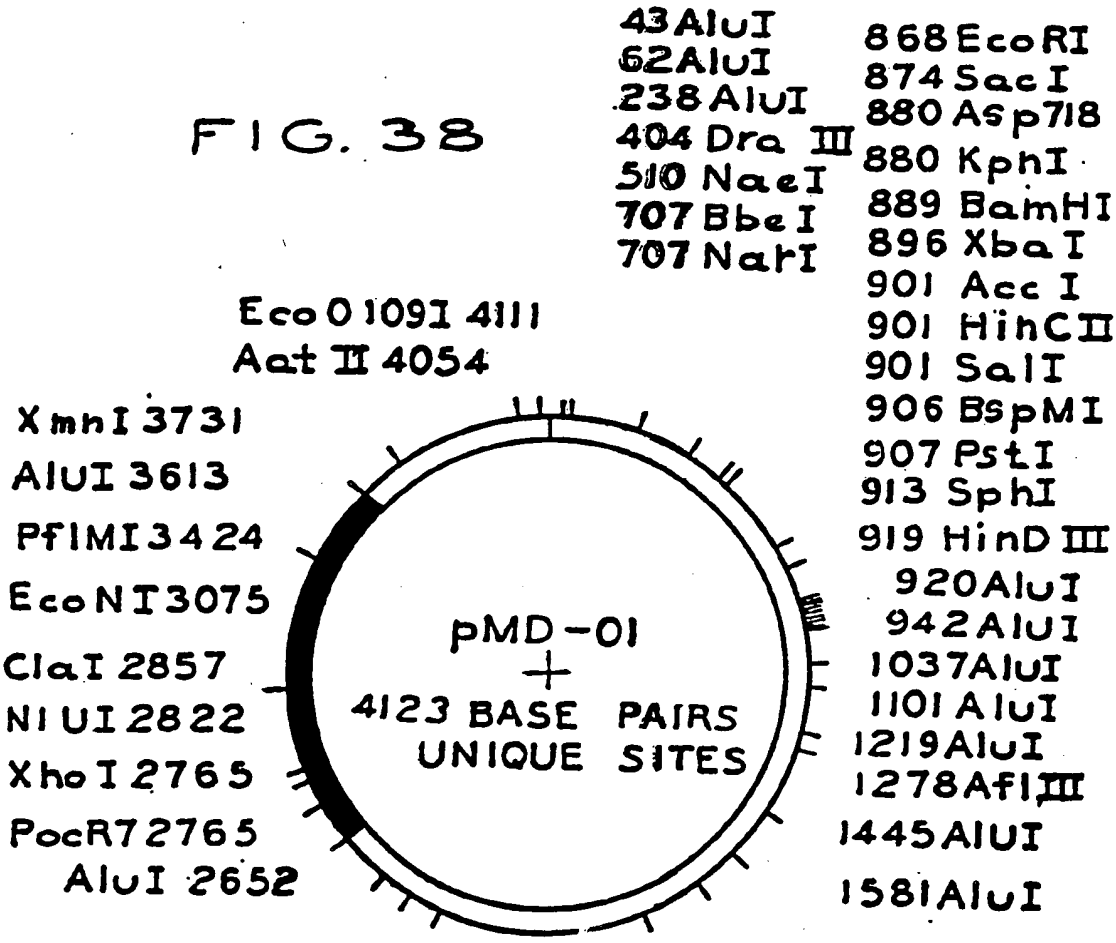
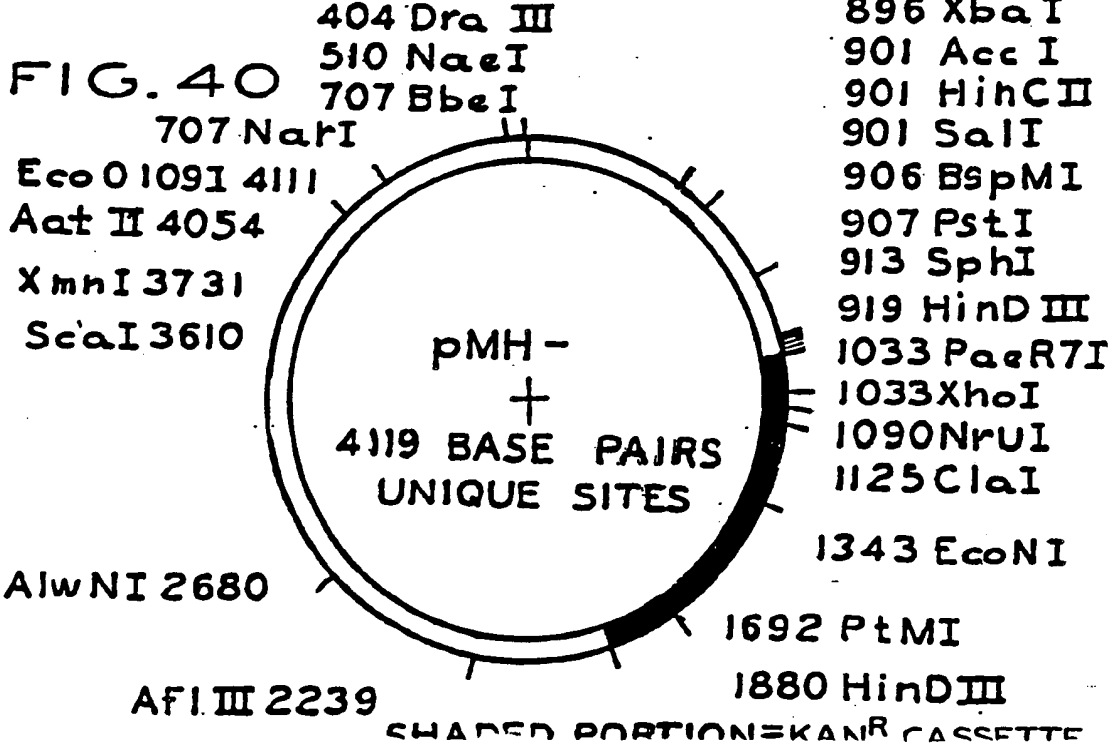

XmnI 8569
707 BbeI
707 NarI
AlwNI 7492
1327 SalI
2075 SalI
2302 NheI
2798 BolI
3044 SalI
3226 SalI
Ex: L5
pMH-5
+
8961 BASE PAIRS
UNIQUE SITES
attP
Int
KanR
HinDIII 6722
PflMI 6534
XmaI 6149
SmaI 6149
XhoI 5875
PaeR7I 5875
HimD III 5761
PstI 5749
SalI 5743
XboI 5737
BamHI 5731
SalI 5310
3839 BamHI
3889 PstI
4324 BSu 36I
AurII 4819
ApaI 4617
SalI 4600

FIG. 38



SHADED PORTION = KAN^R CASSETTE

FIG. 40



SHADED PORTION=KAN^R CASSETTE

FIG. 39

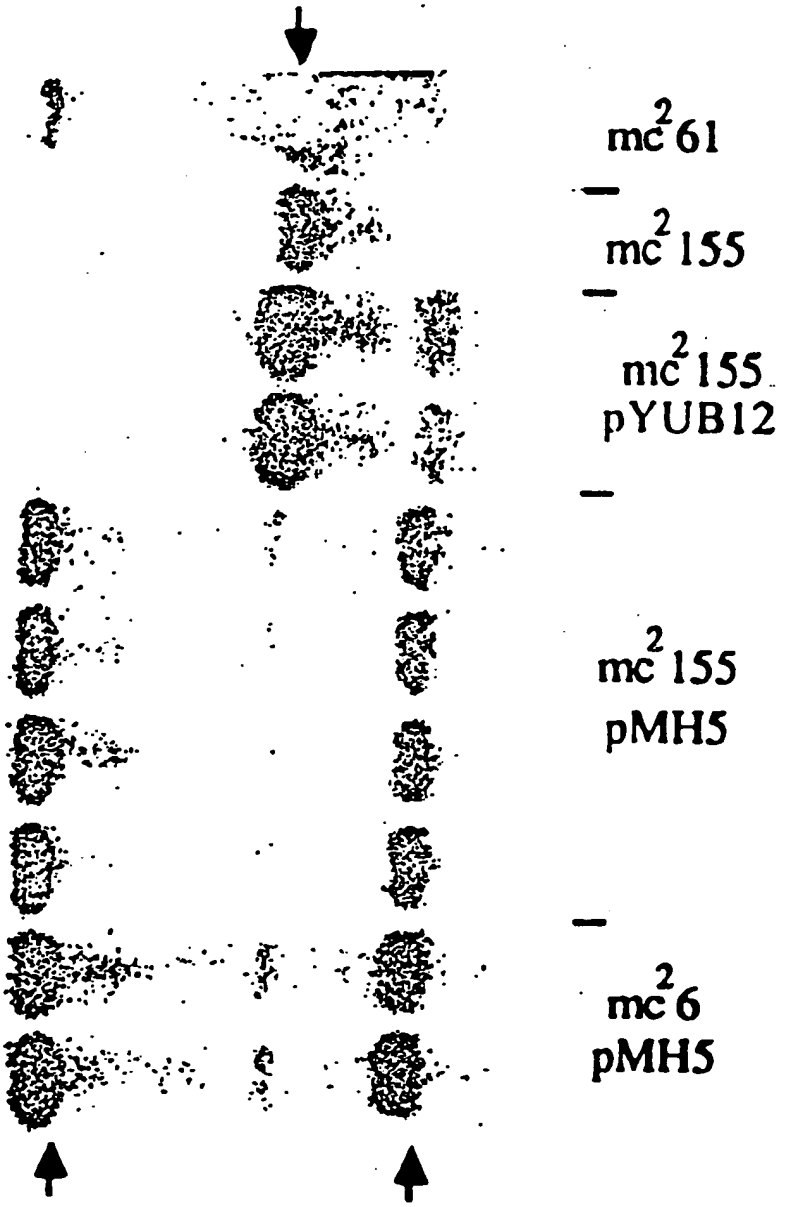


FIG. 41

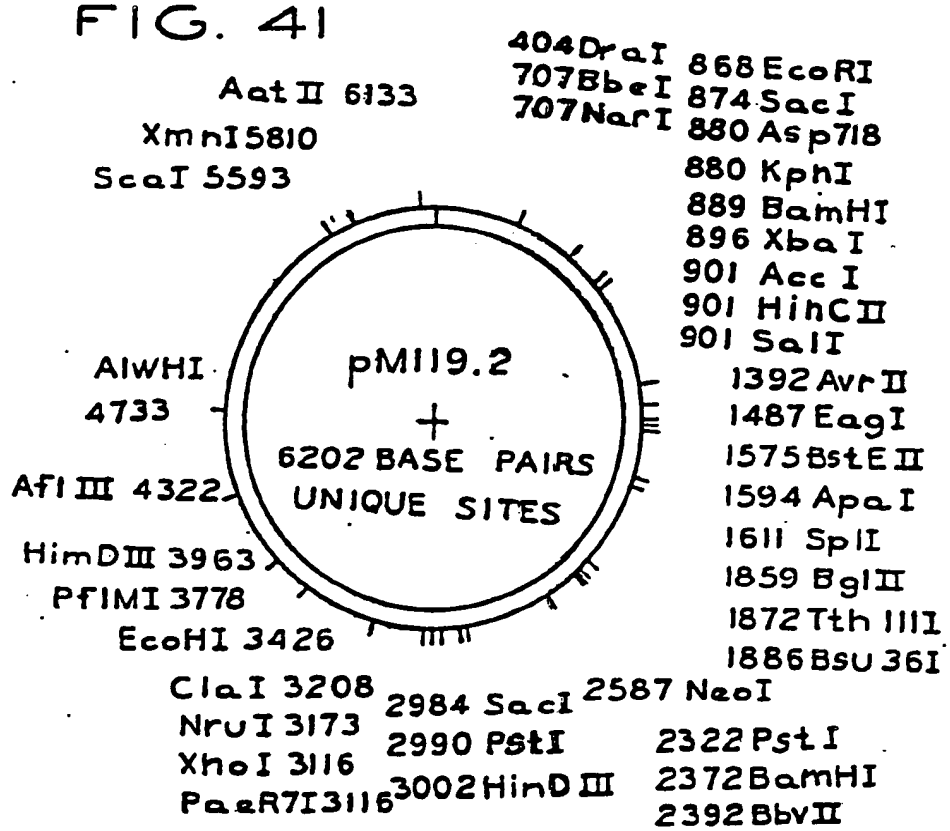

404 DraI
707 BbeI
707 NarI
868 EcoRI
874 SacI
880 Asp718
880 KpnI
889 BamHI
896 XbaI
901 AccI
901 HinCII
901 SalI
1392 AvrII
1487 EagI
1575 BstEII
1594 ApaI
1611 SpeI
1859 BglII
1872 TthIIII
1886 BsU36I
AatII 6133
XmnI 5810
ScaI 5593
AlwHI 4733
AflIII 4322
HimDIII 3963
PflMI 3778
EcoHI 3426
ClaI 3208
NruI 3173
XhoI 3116
PaeR7I 3116
2984 SacI
2990 PstI
3002 HinDIII
2587 NeoI
2322 PstI
2372 BamHI
2392 BbvII
pM119.2
+
6202 BASE PAIRS
UNIQUE SITES

FIG. 42

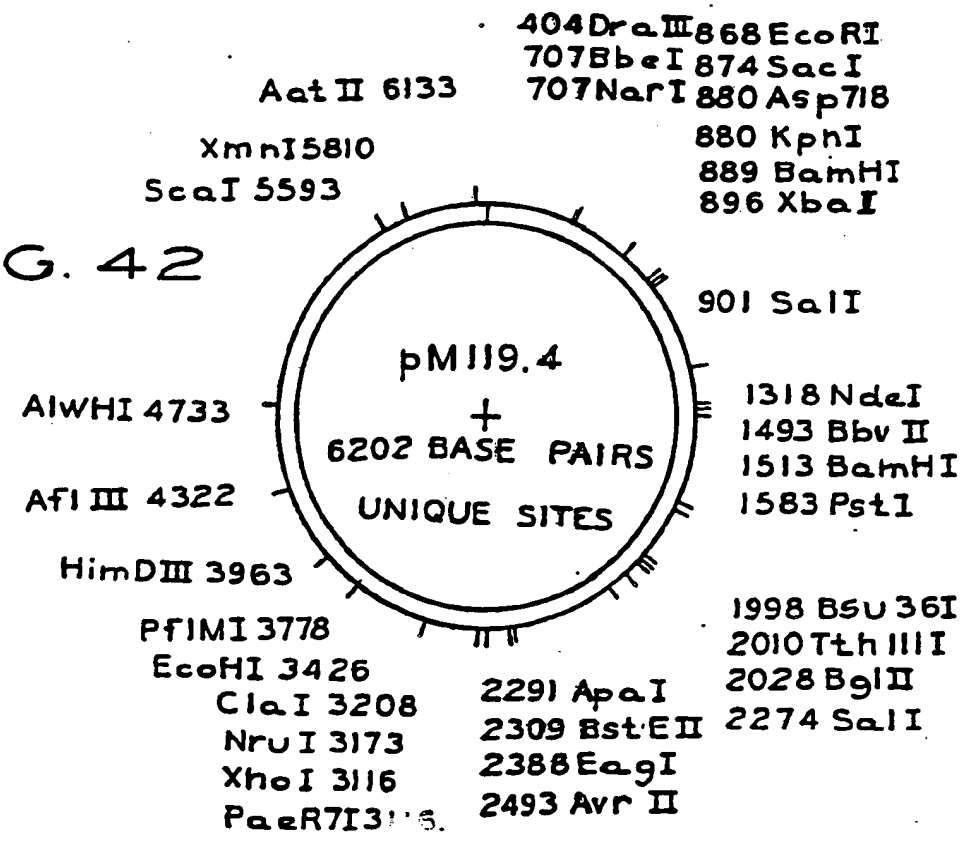

404 DraIII
707 BbeI
707 NarI
868 EcoRI
874 SacI
880 Asp718
880 KpnI
889 BamHI
896 XbaI
901 SalI
1318 NdeI
1493 BbvII
1513 BamHI
1583 PstI
1998 BsU36I
2010 TthIIII
2028 BglII
2274 SalI
2291 ApaI
2309 BstEII
2388 EagI
2493 AvrII
AatII 6133
XmnI 5810
ScaI 5593
AlwHI 4733
AflIII 4322
HimDIII 3963
PflMI 3778
EcoHI 3426
ClaI 3208
NruI 3173
XhoI 3116
PaeR7I 3116
pM119.4
+
6202 BASE PAIRS
UNIQUE SITES

FIG. 43

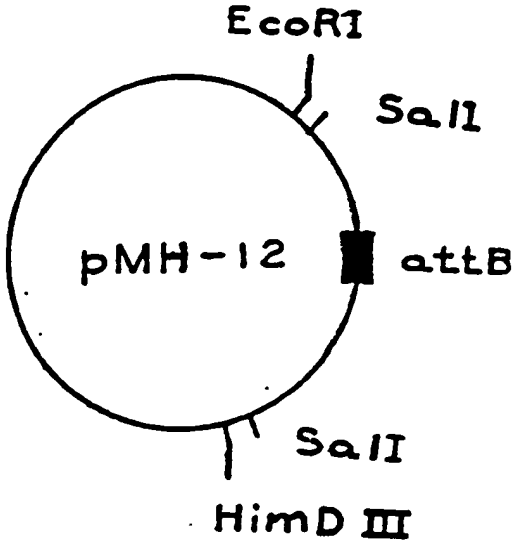

EcoRI
SalI
pMH-12
attB
SalI
HimD III

FIG. 45

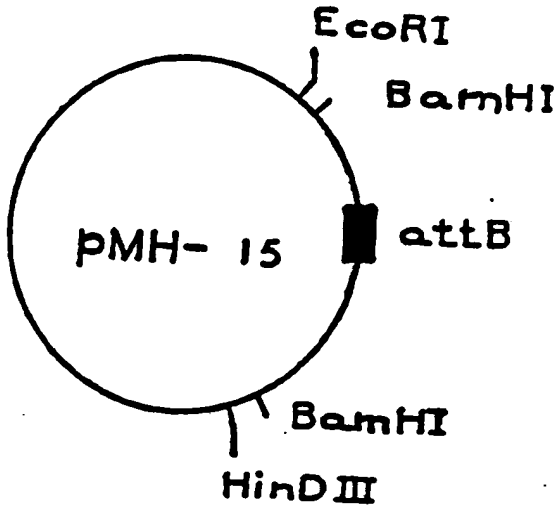
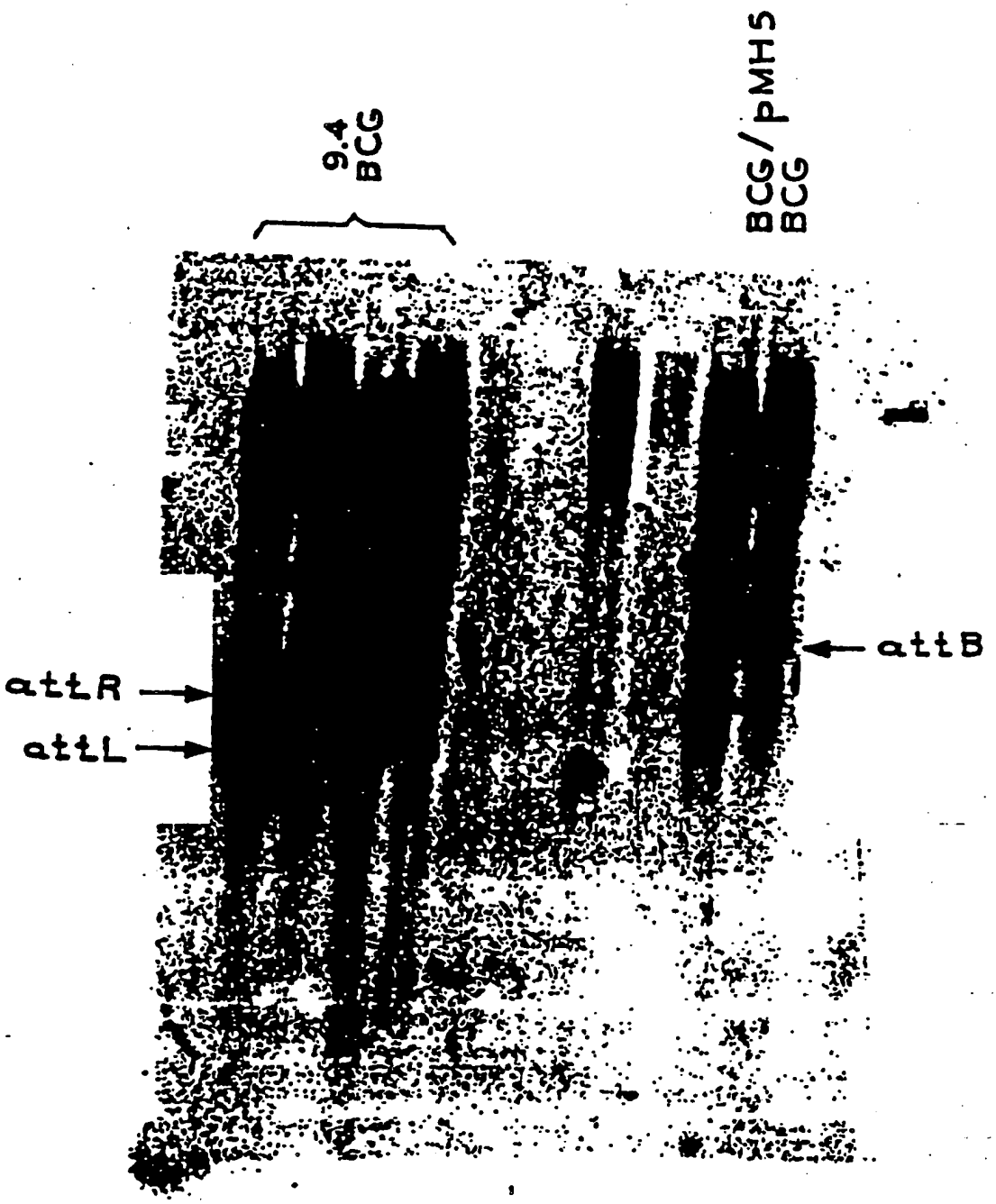

EcoRI
BamHI
pMH-15
attB
BamHI
HinDIII

FIG. 46



51/63

FIG. 47

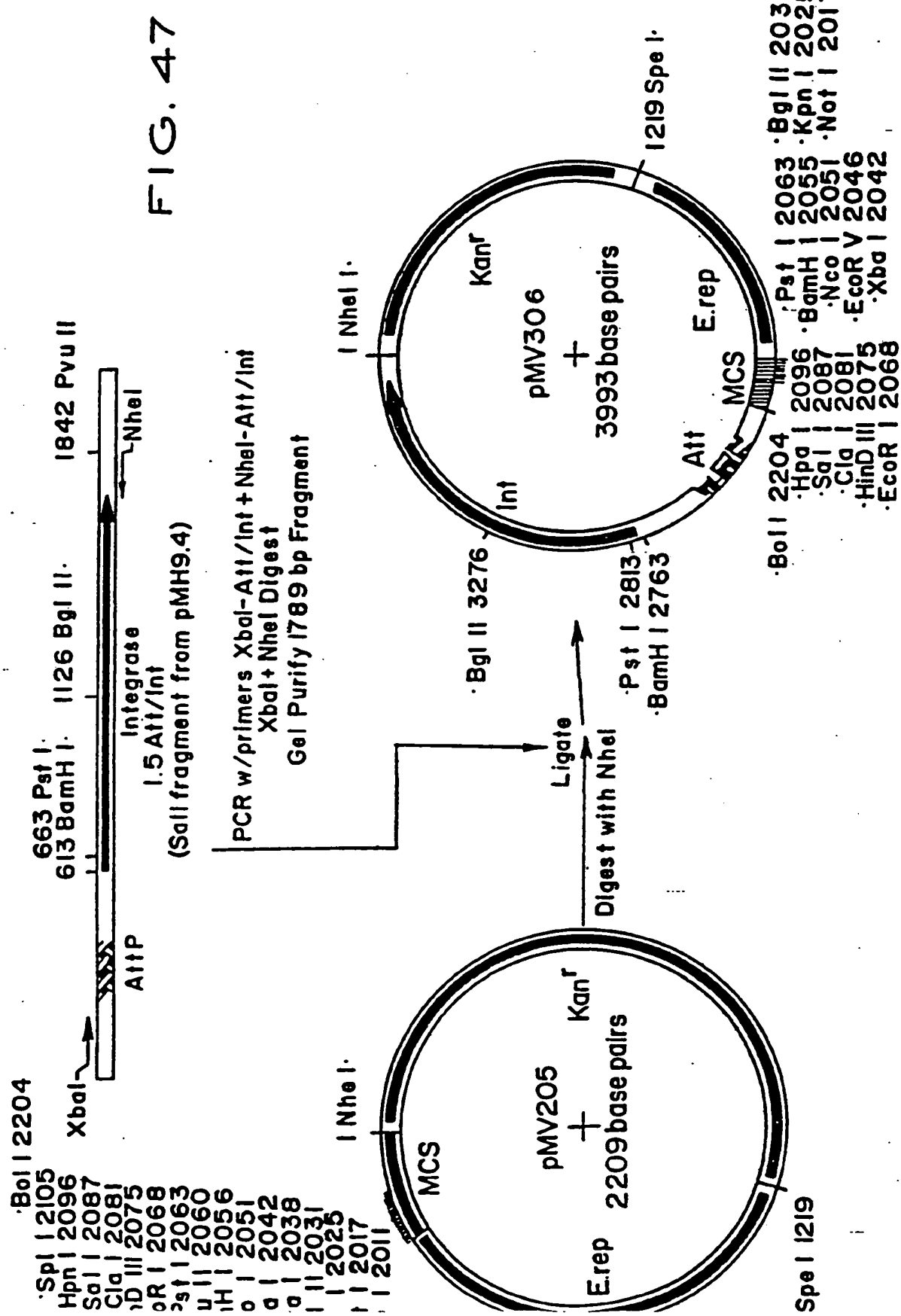
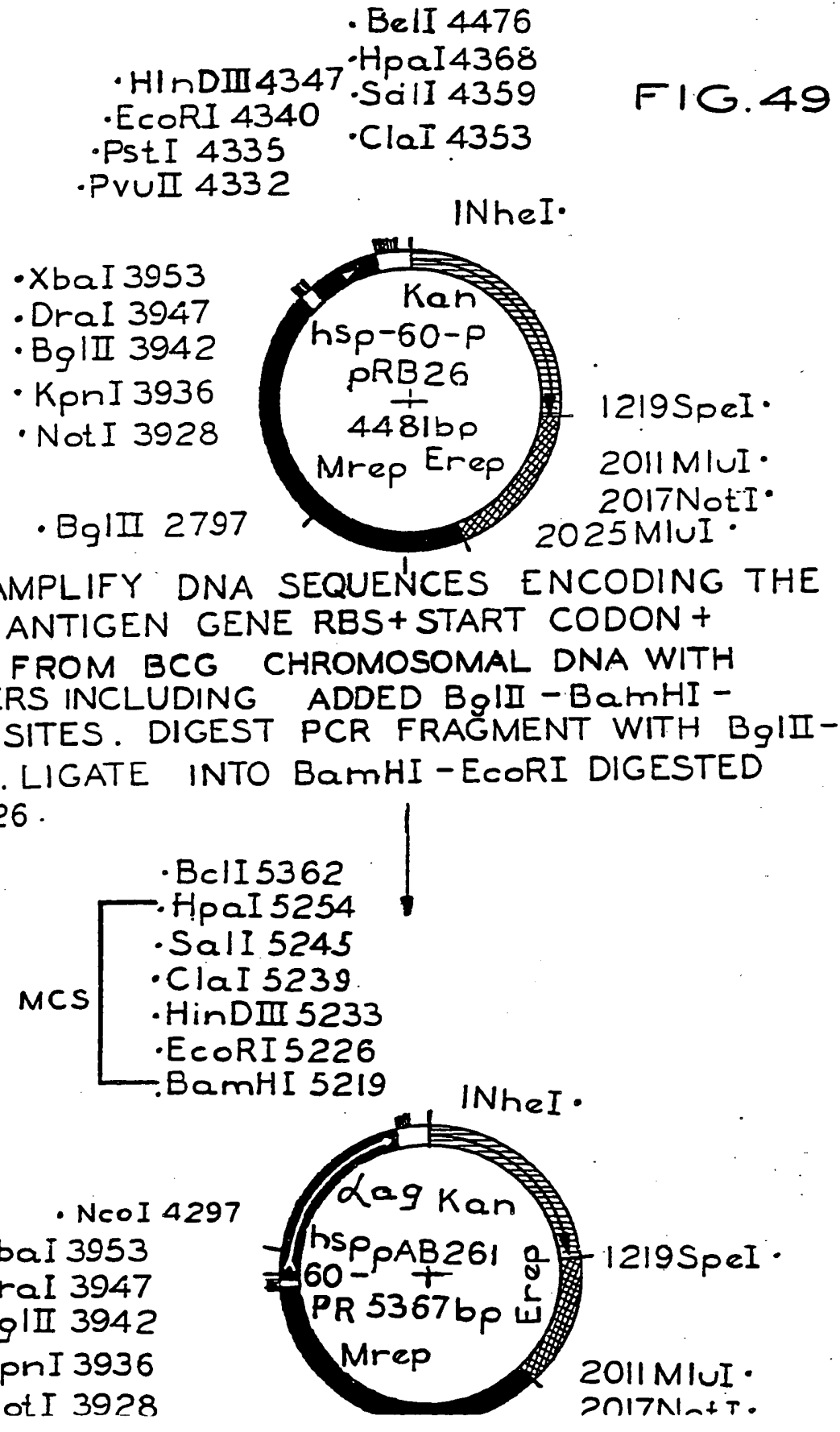

pMV205
+
2209 base pairs
Kanr
MCS
E.rep
Spe I 1219
I Nhe I
Digest with Nhel
Ligate
PCR w/primers XbaI-Att/Int + Nhel-Att/Int
XbaI + Nhel Digest
Gel Purify 1789 bp Fragment
Integrase
1.5 Att/Int
(SalI fragment from pMH9.4)
AttP
XbaI
663 Pst I
613 BamH I
1126 Bgl II
1842 Pvu II
NheI
BclI 2204
SpI I 2105
Hpa I 2096
Sal I 2087
Cla I 2081
HinD III 2075
EcoR I 2068
Pst I 2063
BamH I 2055
Nco I 2051
EcoR V 2046
Xba I 2042
Bgl II 2031
Kpn I 2025
Not I 2017
pMV306
+
3993 base pairs
Kanr
E.rep
MCS
Att
Int
Bgl II 3276
Pst I 2813
BamH I 2763
1219 Spe I
I Nhe I

FIG. 49


·BclI 4476
·HpaI 4368
·HInDIII 4347
·SalI 4359
·EcoRI 4340
·ClaI 4353
·PstI 4335
·PvuII 4332
INheI·
·XbaI 3953
·DraI 3947
·BglII 3942
·KpnI 3936
·NotI 3928
Kan
hsp-60-P
pRB26
+
4481bp
Mrep
Erep
1219SpeI·
2011MluI·
2017NotI·
·BglII 2797
2025MluI·
PCR AMPLIFY DNA SEQUENCES ENCODING THE dag ANTIGEN GENE RBS+START CODON+ GENE FROM BCG CHROMOSOMAL DNA WITH PRIMERS INCLUDING ADDED BglII-BamHI-EcoRI SITES. DIGEST PCR FRAGMENT WITH BglII-EcoRI. LIGATE INTO BamHI-EcoRI DIGESTED P RB 26.
·BclI 5362
·HpaI 5254
·SalI 5245
·ClaI 5239
·HinDIII 5233
·EcoRI 5226
·BamHI 5219
MCS
INheI·
·NcoI 4297
·XbaI 3953
·DraI 3947
·BglII 3942
·KpnI 3936
·NotI 3928
dag Kan
hsp60-
pAB261
+
PR 5367bp
Mrep
Erep
1219SpeI·
2011MluI·
2017NotI·

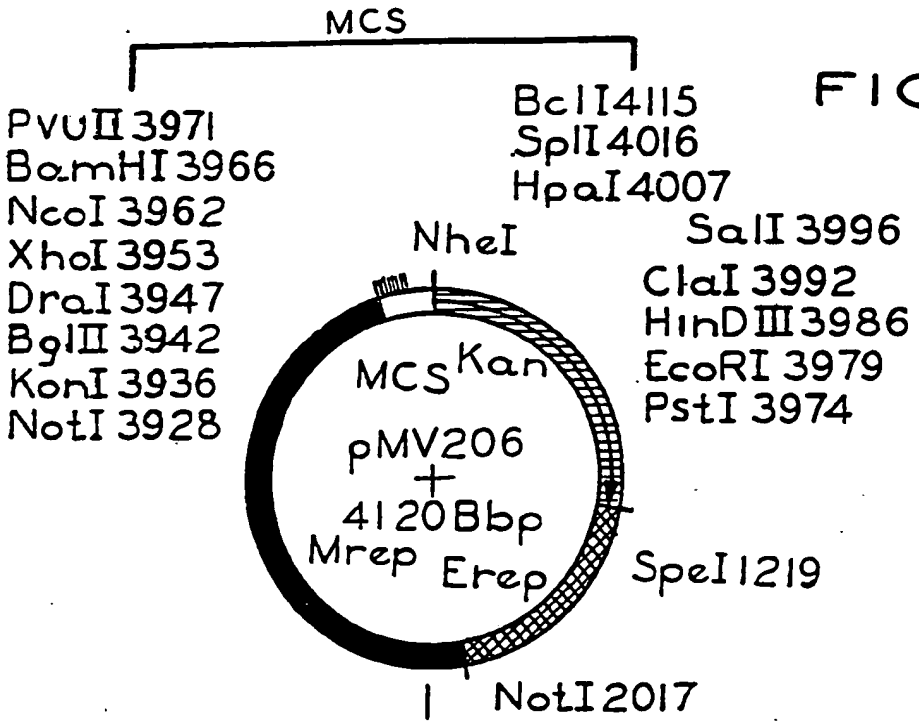


FIG. 51

PCR AMPLIFY hsp70 PROMOTER FROM pMV271 PRIMERS INCLUDING ADDED XbaI-NheI SITES. DIGEST PCRhsp70 FRAGMENT WITH XbaI AND NheI. LIGATE INTO XbaI DIGESTED pMV206 AND SCREEN FOR CORRECT ORIENTATION.

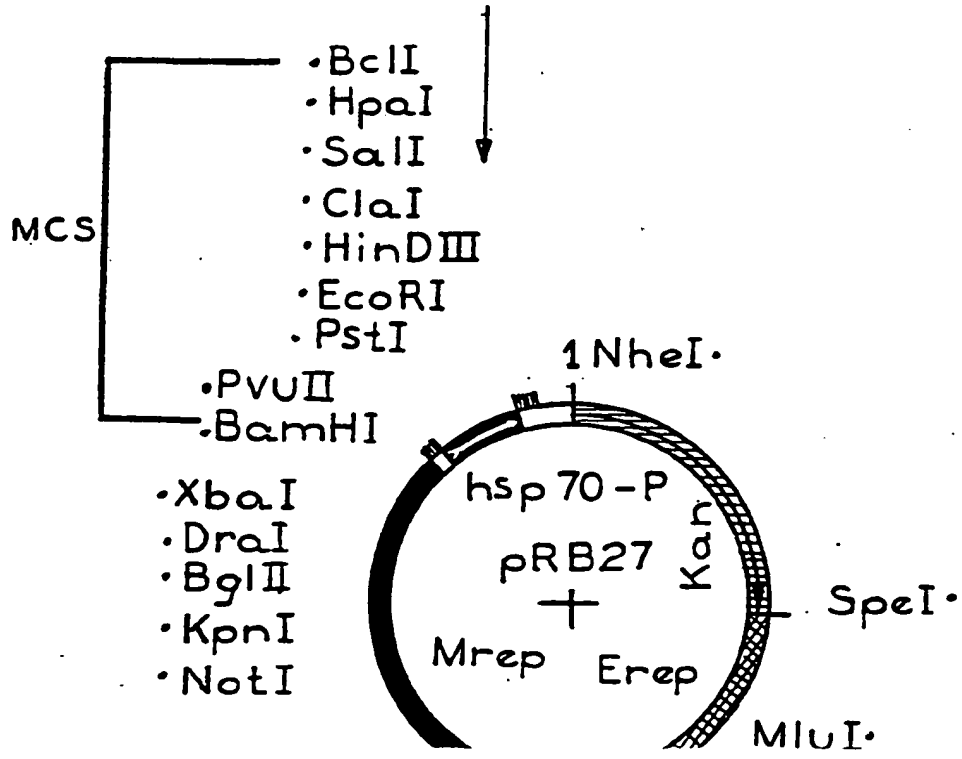
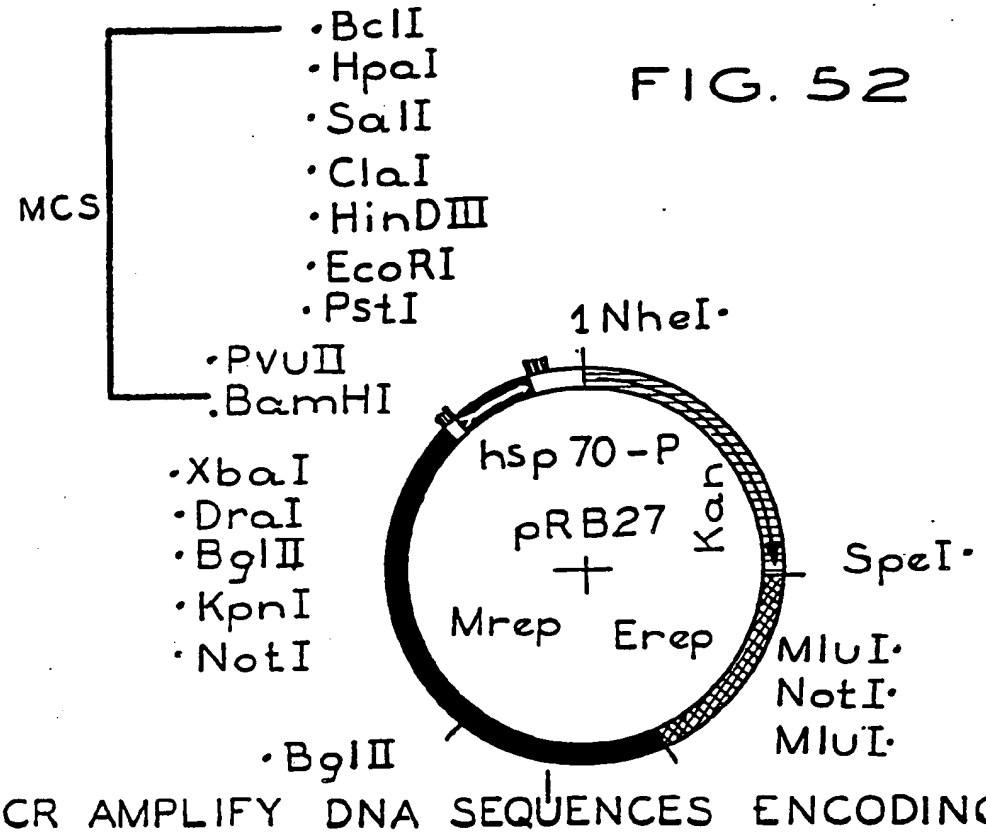


FIG. 52



PCR AMPLIFY DNA SEQUENCES ENCODING THE dag ANTIGEN GENE RBS+START CODON+ GENE FROM BCG CHROMOSOMAL DNA WITH PRIMERS INCLUDING ADDED BglII-BamHI-EcoRI SITES. DIGEST PCR FRAGMENT WITH BglII-EcoRI. LIGATE INTO BamHI-EcoRI DIGESTED P RB 27

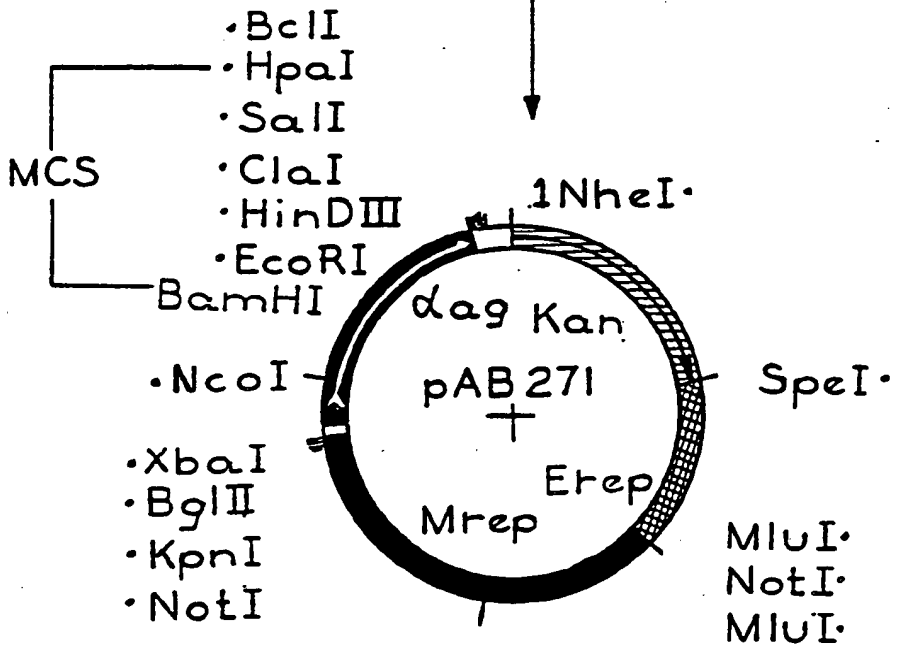
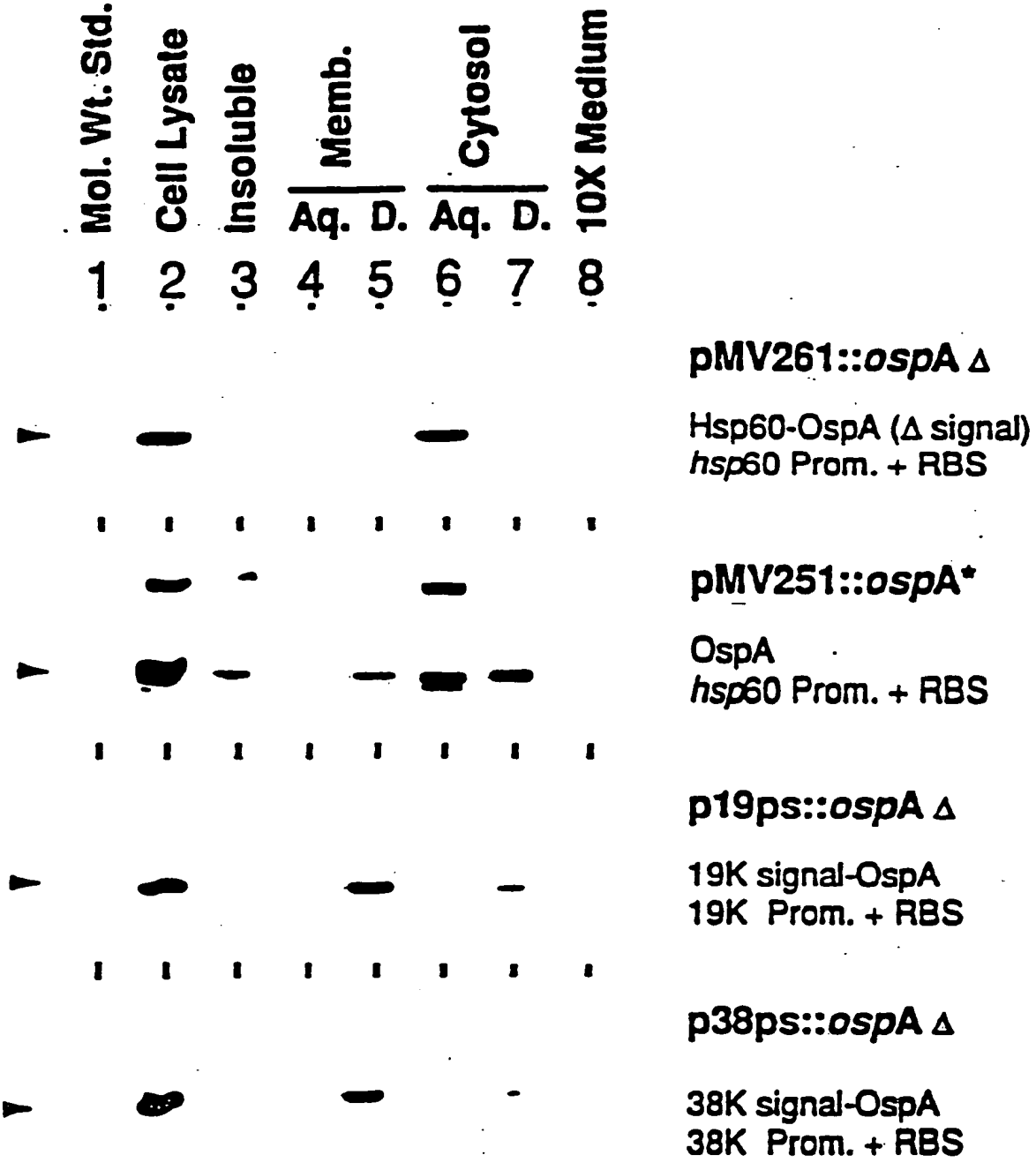


FIG. 53



Triton X-114 Fractionation
SDS-PAGE/Western Blot

FIG. 54

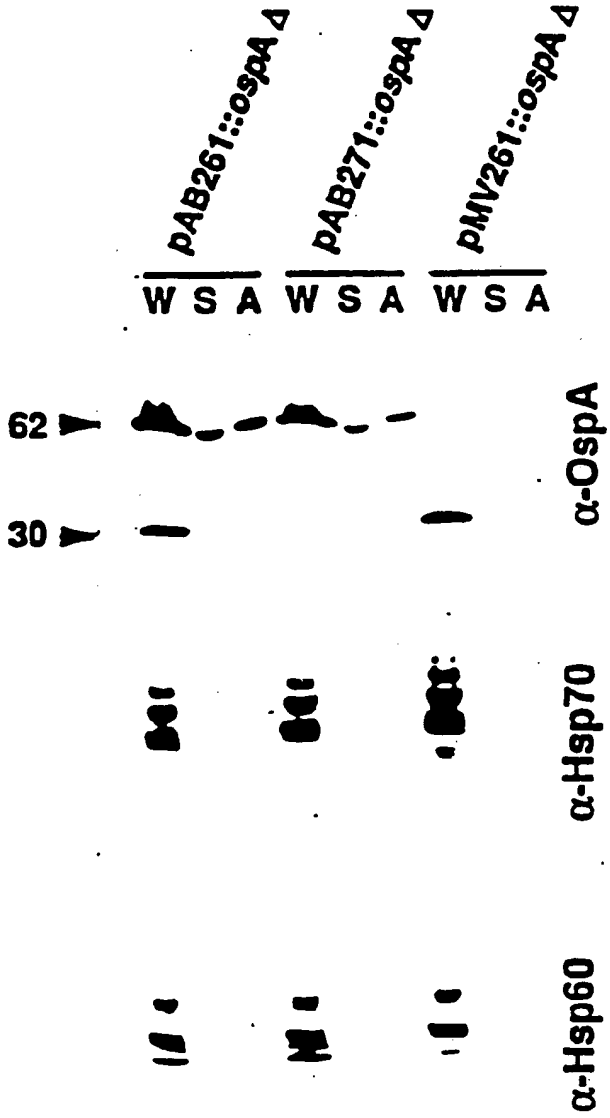

pAB261::ospA Δ
pAB271::ospA Δ
pMV261::ospA Δ
W S A W S A W S A
62
30
α-OspA
α-Hsp70
α-Hsp60

FIG. 55

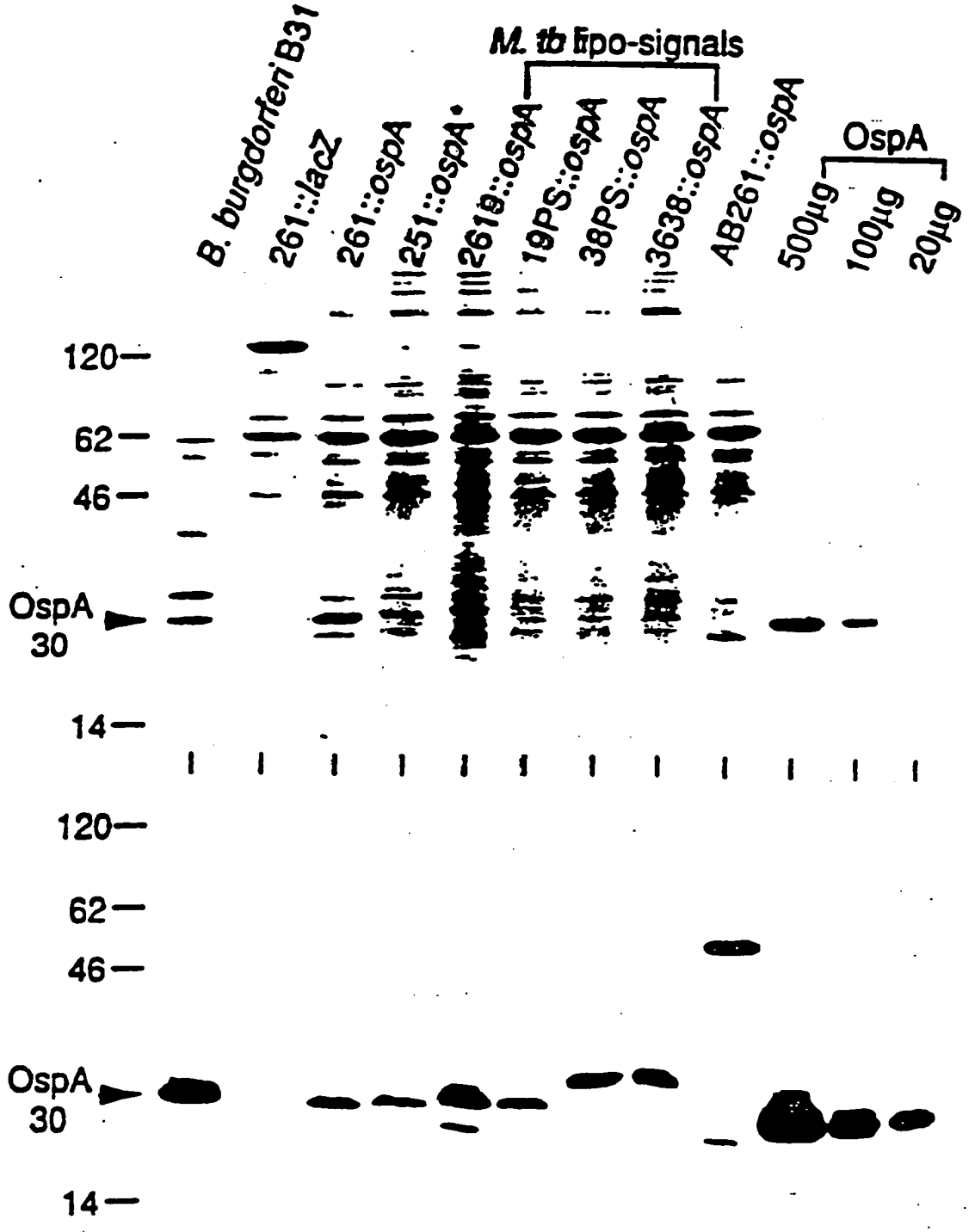

B. burgdorferi B31
261::lacZ
261::ospA
251::ospA
2618::ospA
19PS::ospA
38PS::ospA
3638::ospA
AB261::ospA
M. tb lipo-signals
OspA
500µg
100µg
20µg
120
62
46
OspA 30
14
120
62
46
OspA 30
14

FIG. 48

```
        1   10        1   20        1   30        1   40        1   50        1   60
  1 AGATCTGAGC ACACGACGAC ATACAGGACA AAGGGGCACA GGTATGACAG ACGTGAGCCG 60
 61 AAAGATTCGA GCTTGGGGAC GCCGATTGAT GATCGGCACG GCAGCGGCTG TAGTCCTTCC 120
121 GGGCCTGGTG GGGCTTGCCG GCGGAGCGGC AACCGCGGGC GCGTTCTCCC GGCCGGGGCT 180
181 GCCGGTCGAG TACCTGCAGG TGCCGTCGCC GTCGATGGGC CGCGACATCA AGGTTCAGTT 240
241 CCAGAGCGGT GGGAACAACT CACCTGCGGT TTATCTGCTC GACGGCCTGC GCGCCCAAGA 300
301 CGACTACAAC GGCTGGGATA TCAACACCCC GGCGTTCGAG TGGTACTACC AGTCGGGACT 360
361 GTCGATAGTC ATGCCGGTCG GCGGGCAGTC CAGCTTCTAC AGCGACTGGT ACAGCCCGGC 420
        1   10        1   20        1   30        1   40        1   50        1   60
```


FIG. 50

```
        1   10        1   20        1   30        1   40        1   50        1   60
  1 TCTAGACCCG CACGACCAGC GTTAGCATGC TCAGTAAGTT GAGTGCATCA GGCTCAGCTC 60
 61 TCAATTGACA GCACACCGCC GTCGAGGCAA GCTTGAGCGG GGTGCACTCA TCATAGCTAG 120
121 C                                                                 121
        1   10        1   20        1   30        1   40        1   50        1   60
```

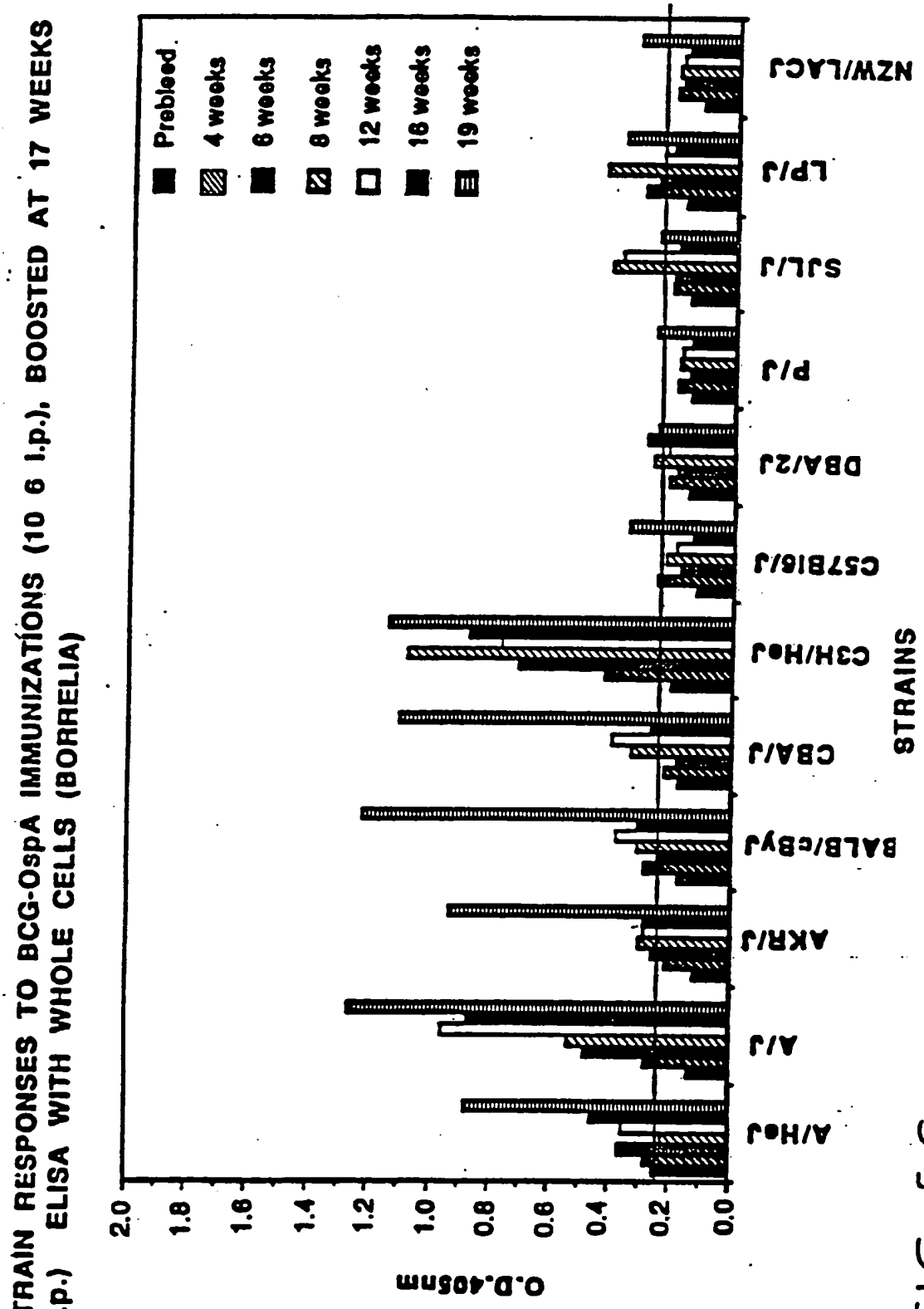

MICE STRAIN RESPONSES TO BCG-OspA IMMUNIZATIONS (10 6 I.p.), BOOSTED AT 17 WEEKS
(10 6 I.p.) ELISA WITH WHOLE CELLS (BORRELIA)
Prebled
4 weeks
6 weeks
8 weeks
12 weeks
16 weeks
19 weeks
O.D.405nm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A/HeJ
A/J
AKR/J
BALB/cByJ
CBA/J
C3H/HeJ
C57BI6/J
DBA/2J
P/J
SJL/J
LP/J
NZW/LACJ
STRAINS

FIG. 56

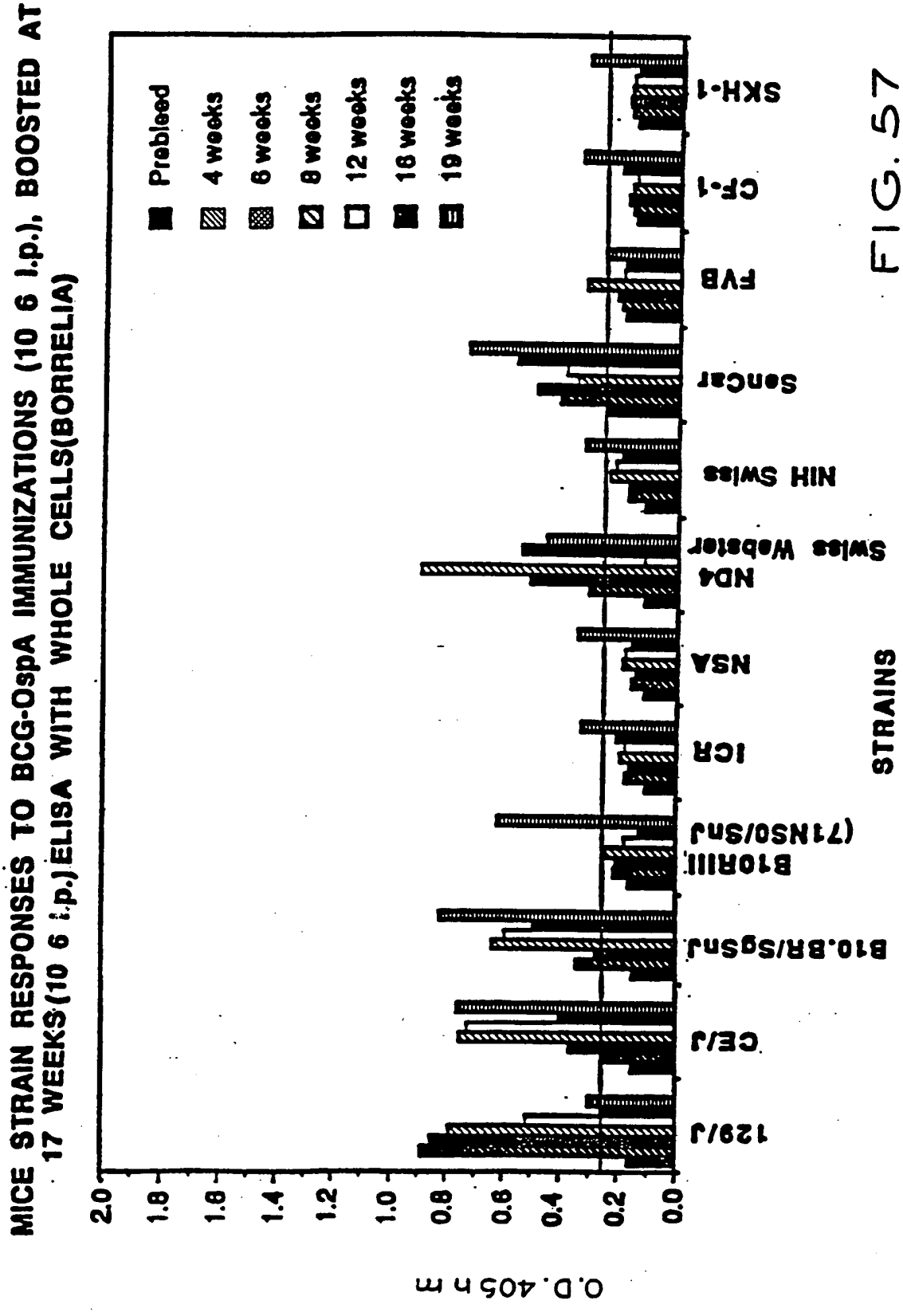

MICE STRAIN RESPONSES TO BCG-OspA IMMUNIZATIONS (10 6 i.p.), BOOSTED AT 17 WEEKS(10 6 i.p.)ELISA WITH WHOLE CELLS(BORRELIA)
Prebleed
4 weeks
6 weeks
8 weeks
12 weeks
16 weeks
19 weeks
O.D.405nm
2.0
1.8
1.8
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
129/J
CE/J
B10.BR/SgSnJ
B10RIII
(71NSO/SnJ
ICR
NSA
NO4
Swiss Webster
NIH Swiss
Sencar
FVB
CF-1
SKH-1
STRAINS

FIG. 57

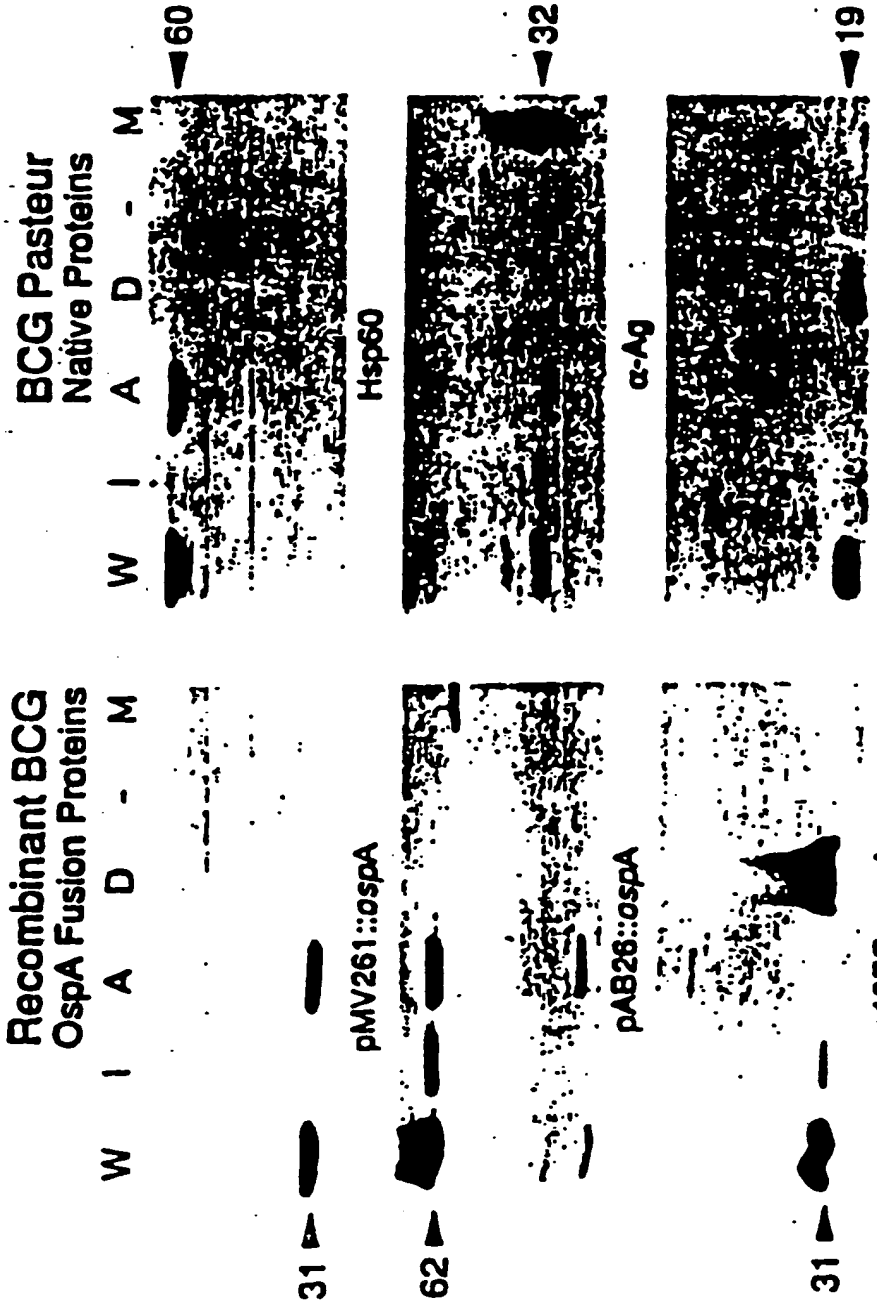

BCG Pasteur
Native Proteins
W I A D - M
60
32
19
Hsp60
α-Ag
Mtb19
Recombinant BCG
OspA Fusion Proteins
W I A D - M
pMV261::ospA
pAB28::ospA
p19PS::ospA
pMV251::ospA
31
62
31
31

FIG. 58

FIG. 59

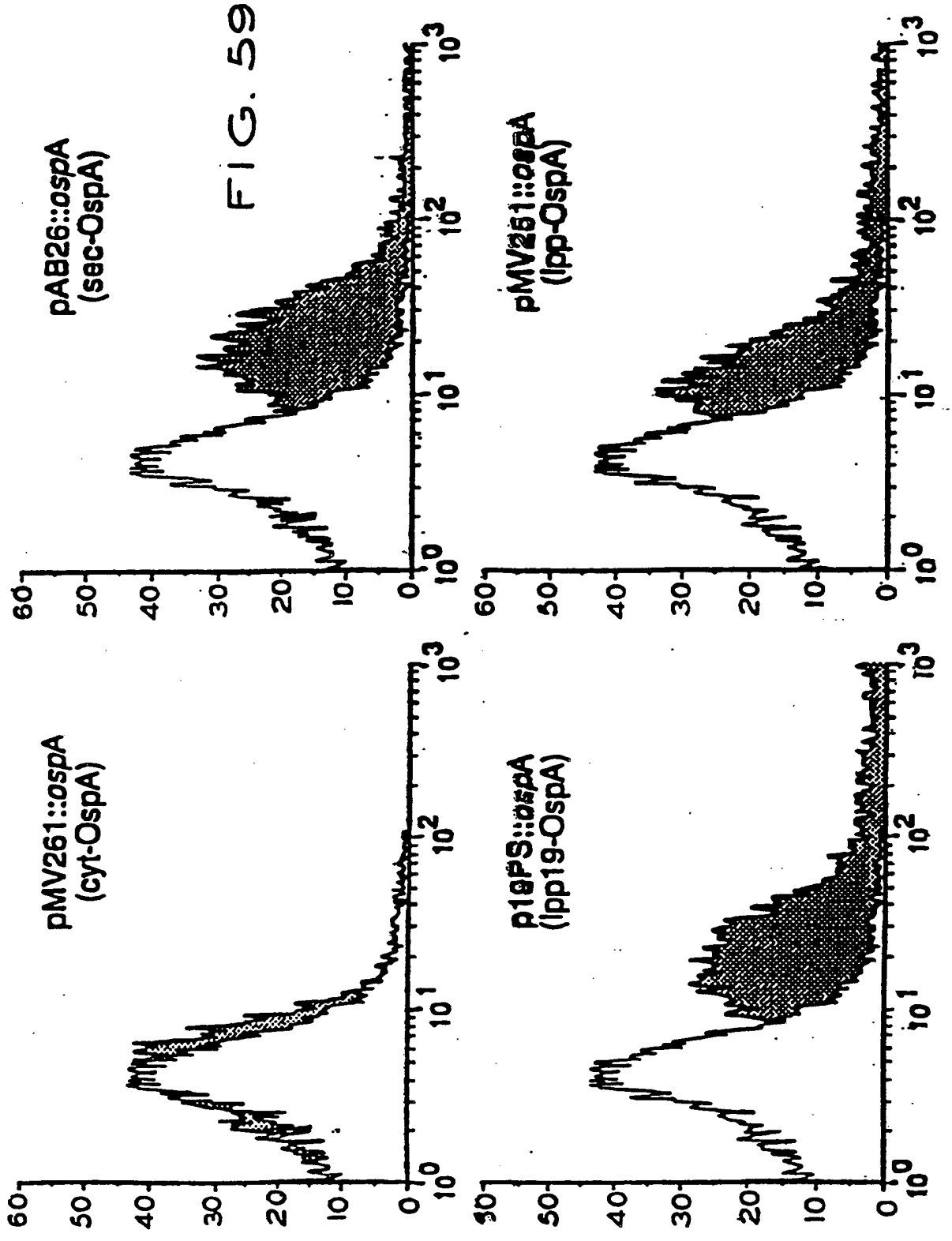
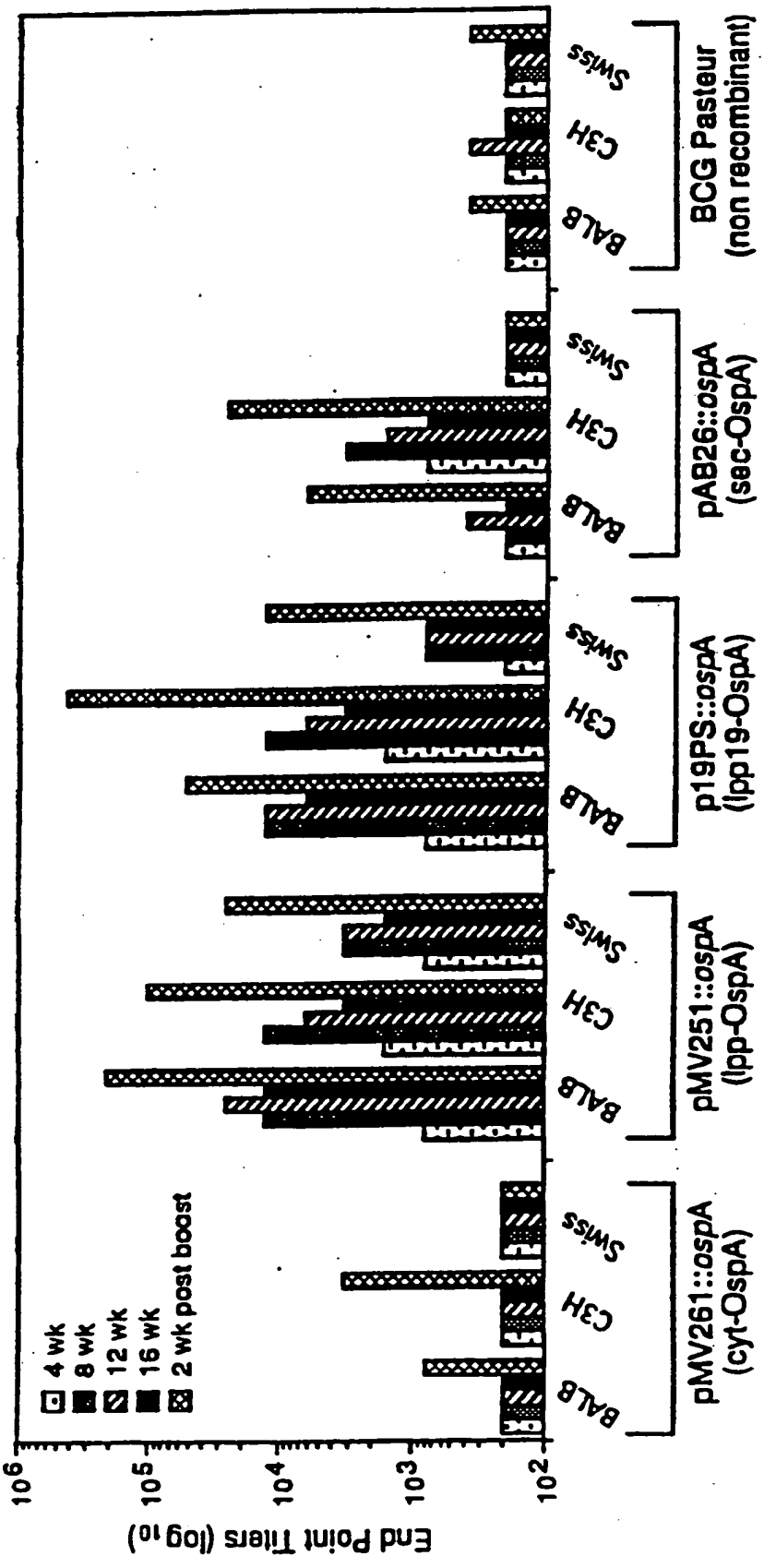

pAB26::ospA
(sec-OspA)
pMV261::ospA
(lpp-OspA)
pMV261::ospA
(cyt-OspA)
p19PS::ospA
(lpp19-OspA)
60
50
40
30
20
10
0
10^0
10^1
10^2
10^3

FIG. 6O


End Point Titers (log_{10})
10^6
10^5
10^4
10^3
10^2
4 wk
8 wk
12 wk
16 wk
2 wk post boost
pMV261::ospA (cyt-OspA)
pMV251::ospA (lpp-OspA)
p19PS::ospA (lpp19-OspA)
pAB26::ospA (sec-OspA)
BCG Pasteur (non recombinant)
BALB
C3H
Swiss

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09075

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/04; C12N 15/74, 1/21
US CL :435/320.1, 252.3; 424/93
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 252.3; 424/93

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Biosis, World Patents Index.
Search terms:signal sequence, secretion sequence, mycobacteria, lipoprotein, Streptococcus pneumoniae, pspA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nucleic Acids Research, Vol. 17, No. 21, issued 1989, Wallich et al., "Cloning and sequencing of the gene encoding the outer surface protein A (OspA) of a European Borrelia burgdorferi isolate", page 8864. See entire article.	14-21
P,Y	Journal of Bacteriology, Vol. 174, No. 2, issued January 1992, Yother et al., "Structural properties and evolutionary relationships of PspA, a surface protein of Streptococcus pneumoniae, as revealed by sequence analysis", pages 601-609. See entire article.	8
Y	Nucleic Acids Research, Vol. 17, No. 3, issued 1989, Ashbridge et al., "Nucleotide sequence of the 19 kDa antigen gene from Mycobacterium tuberculosis", page 1249. See entire article.	1-13, 22
Y	Gene, Vol. 71, issued 1988, Rauzier et al., "Complete nucleotide sequence of pAL5000, a plasmid from Mycobacterium fortuitum", pages 315-321. See entire article.	1-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 January 1993	Date of mailing of the international search report 26 JAN 1993
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer PHILIP CARTER Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09075

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Infection and Immunity, Vol. 57, No. 8, issued August 1989, Andersen et al., "Structure and mapping of antigenic domains of protein antigen b, a 38,000-molecular-weight protein of Mycobacterium tuberculosis", pages 2481-2488. See entire article.	1-13, 22
Y	Proceedings of the National Academy of Sciences USA, Vol. 85, issued September 1988, Snapper et al., "Lysogeny and transformation in mycobacteria: Stable expression of foreign genes", pages 6987-6991. See entire article.	7
Y	Nature, Vol. 351, issued 06 June 1991, Stover et al., "New use of BCG for recombinant vaccines", pages 456-460. See entire article.	1-22